Synergy of Repression and Silencing Gradients along the Chromsome in Saccharomyces cerevisiae

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1. ABSTRACT

The interplay of transcriptional activator and repressors bound to the promoter regulates gene expression. We corroborate in our work that these repressors form gradients across the chromosome and counteract the gene activation. Ssn6, which a promoter-specific global repressor, generates inhibitory gradients with similar slopes as Sir3, which is a regional silencer. With increase in distance between the promoter and the inhibitor recruiting sequences, the fold inhibition declines similarly with Ssn6 and Sir3. When the reporter gene is flanked by two repressor (Ssn6) gradients, they interact multiplicatively. When two silencing gradients flank the reporter gene, the gradients interact synergistically and the effect on gene expression is much stronger than in the case of repression gradients. Two silencing gradients flanking the reporter gene also give rise to variegated expression, when the nucleation widths reach a certain threshold. The architecture of the nucleation sites also determined the strength of silencing and variegated expression. The quantification of these inhibitory gradients across the chromosome helps to understand the co-expression pattern of adjacent genes in the genome, tissue engineering and cellular differentiation.

2. ZUSAMMENFASSUNG

Genexpression wird durch das Zusammenspiel von Transkriptionsaktivatoren und –repressoren, welche an den Promotor binden, reguliert. Wir bestätigen in unserer Arbeit, dass Repressoren entlang des Chromosoms Gradienten bilden und der Genaktivation entgegen wirken. Ssn6, ein Promotor spezifischer Repressor, bildet inhibitorische Gradienten mit ähnlicher Steigung wie das örtlich begrenzte Genstillegungsprotein Sir3 (Silencer). Mit größer werdender Distanz zwischen Promotor und Inhibitor rekrutierenden Sequenzen, sinkt die Inhibition in ähnlicher Weise bei Ssn6 und Sir3. Wenn das Reportergen mit zwei Repressoren (Ssn6) Gradienten flankiert ist, interagieren diese Gradienten muliplikativ. Wenn zwei genstilllegungs Gradienten das Reporter Gen flankieren, interagieren die Gradienten synergistisch und sie haben einen viel stärkeren Effekt auf die Genexpression als im Falle der Repressionsgradienten. Zwei genstilllegungs Gradienten, welche das Reporter Gen flankieren, können vielfältige Expression zur Folge haben, falls die Nukleierungsbreite einen gewissen Schwellenwert erreicht. Die Architektur der Nukleierungsstelle bestimmt den Hemmungswirkungsgrad und die vielfältige Expression. Mit Hilfe der Quantifizierung dieser inhibitorischen Gradienten entlang des Chromosoms kann man das Coexpressionsmuster von anliegenden Genen im Genom besser verstehen und diese Einsichten können auch in Tissue Engineering und in Zell differenzierung experimenten angewendet werden.

3. INTRODUCTION

Saccharomyces cerevisiae is a single-celled eukaryote. It counters environmental changes by altering gene expression. Many cellular processes are conserved from yeast to human. About 220 different cell types of human are known to be specialized for particular functions. Though they are comprised of the same genome, their functional differences can attribute to patterns of gene expression. Cell differentiation is a complex process and it requires precise control mechanisms that allow expression of appropriate subset of genes and repression of genes specific to other cell type. Transcriptional regulation coordinates different processes that occur in the cell. Whether it is cell differentiation, cell growth, cell cycle, metabolic pathways, response to environmental changes, *etc.* all the processes are regulated at the genetic level. *Saccharomyces cerevisiae* provides a simple and powerful system to analyze this complex process genetically in living cells and this work typically translates directly to more-complex eukaryotic systems where mechanisms of transcription and its regulation are largely conserved.

Like higher Eukaryotes, *Saccharomyces cerevisiae* has three RNA Polymerases. RNA pol I is involved in the transcription of ribosomal DNA. RNA pol II is responsible for the transcription of protein encoding genes. RNA pol III transcribes ribosomal 5s RNA, tRNA, catalytic or structural RNA molecules, which are involved in protein synthesis, pre-mRNA splicing, tRNA processing, and the control of RNA polymerase II elongation. The composition and architecture of general transcription factors and the mediator complexes are very similar in yeast and humans. In *Saccharomyces cerevisiae,* transcription is initiated at promoters of protein encoding genes, by RNA pol II and a number of auxiliary complexes with a multitude of subunits. These complexes are general transcription factors (GTF), mediator (Srb/Med) complexes and gene specific transcription factors. These gene specific transcription factors recruit chromatin remodeling complexes. The implications of these chromatin modifiers on the dynamic matrix of chromatin structure and in turn regulation of transcription are a major area of research.

3.1 Regulation of gene expression

The regulation of gene expression starts at the level of nucleosomes. Nucleosomes are the fundamental repetitive structures that wrap the DNA around themselves and form the chromatin. Nucleosome is an octamer core made up of histone proteins two each of H2A, H2B, H3 and H4 and one molecule of H1 which directs the wrapping of 147 bp of DNA around the histone octamer. The core histones are basic in nature and are made up of Arg and Lys. Post-translational modification of these amino acids regulates loosening and compacting of the DNA. His, Ser and Thr residues are also subject to modifications. Histones can be acetylated, phosphorylated, methylated, ubiquitylated, sumoylated and ribosylated. The N-terminal tails of H3 and H4 are rich in Arg and Lys which can be acetylated reducing the positive charge of the histones. N-terminal ends provide inter-nucleosome contacts, acetylation destabilizes chromatin packing.

Histone	Amino Acids
H2A	S1, K5, K9, K119
H2B	K5, K12, S14, K15, K20, K120
H3	R2, T3, R8, K9, S10, K14, R17, K18, K23, R26, K27, S28, K36, K56, K79
H4	S1, R3, K5, K8, K12, K16, K20

Table 1: Amino acids of histones that undergo various modifications.

Gene specific transcription factors are activators and repressors. These factors bind to the upstream activating sequences (UAS) and upstream repressor site (URS) respectively. Activators are DNA-binding proteins that enhance transcription. These activators interact with co-activators. Co-activators in turn interact with transcription machinery and stimulate initiation or elongation of transcription. They recruit histone modifiers to the promoter leading to chromatin remodeling, which enhances the accessibility of the DNA to the transcription machinery. Repressors hinder transcription. They interact with co-repressors which compact the DNA and make it less available for the transcription apparatus. These co-repressors in turn recruit histone remodelers which organize the chromatin into a repressive structure. The reciprocity of the co-activators and co-repressors regulates the balance between active and repressed chromatin. Gene expression can be inhibited by Silent Information Regulators (Sir2, Sir3 and Sir4) also in

budding yeast. Silencing is a promoter independent phenomenon that spans a chromosomal domain aiming nucleosomes. It is observed at telomeres, mating type loci and rDNA.

3.2 Repression: Ssn6-Tup1

Ssn6-Tup1 is a global corepressor required for establishing and maintaining the repression of varied set of genes. Ssn6 and Tup1 exist as a complex and are first identified as proteins involved in the glucose repression (Trumbly RJ, J. Bacteriol 1986; Wiliams *et al*., Mol. Cell. Biol. 1991). It is one of the first corepressor complexes to be identified and it is involved in the repression of over 180 genes in *Saccharomyces cerevisiae*. Sequence-specific DNA binding repressors determine the specificity of repression of Ssn6-Tup1. Some of such repressors are Mig1 (glucose-repressible genes), Ctr1 (DNA damage response genes), α2 repressor (a-cell specific genes) and Rox1 (hypoxic genes).

Ssn6 was isolated as a suppressor for the necessity of SNF1 kinase for *SUC2* gene expression (Carlson M, *et al*, Genetics, 1984). Ssn6 is a TPR (tetratricopeptide repeat) motif protein. Similar repeats are in Cdc16 and Cdc23. The TPR sequences are highly conserved amphipathic α-helices which mediate protein-protein interactions (Sikorski RS, Cell 1990). Ssn6 has ten TPR motifs at the N-terminus. TPR domains 1-3 interact with Tup1 and the rest mediate recruitment of Ssn6-Tup1 to different promoters. C-terminus of Ssn6 can be phosphorylated (Schultz *et al*., Mol. Cell. Biol. 1990).

Tup1 is a WD40 or beta-transducin motif protein that has highly conserved tryptophanyl and aspartyl residues positioned at every 40 residues. N-terminus (first 70 amino acids) is involved in multimerization and interacts with Ssn6. Trimer or tetramer of Tup1 associates with Ssn6 (Redd *et al*., J. Biol. Chem 1997; Varanasi *et al*., Mol. Cell. Biol. 1996). The region of Tup1 from 73rd to 386th amino acids interacts with H3 and H4. (Edmondson DG, Genes Dev. 1996). C-terminus has WD repeats and is involved in protein-protein interaction. Tup1 interacts with the α2 repressor (Komachi *et al.,* Genes Dev.1994).

The complex binds to repressors and leads to gene repression. When Tup1 is targeted to the promoter it can cause repression even in the absence of Ssn6 (Tzamarias and Struhl, Nature 1994.). When brought to the promoter Ssn6 can repress gene expression, only in the presence of

Tup1. Ssn6 interacts with certain repressors like α2 (Smith RL *et al.*, Genes Dev. 1995) and Crt1 (Huang M, Cell 1998). Through these interactions repressors recruit co-repressors to the site of requirement. Ssn6 is required to recruit Tup1 to the promoter that is to be repressed (Keleher *et al.*, Cell 1992). In Ssn6 mutant strains Tup1 expression is not effected but recruitment is drastically reduced (Judith K. Davie *et al.*, Mol. Cell. Biol. 2002).

Ssn6-Tup1 is involved in repressing varied group of genes. The complex represses genes involved in glucose metabolism, osmosis, hypoxia, DNA damage and genes that determine mating type. The common aspects in all genes are histones and basal transcription machinery, which could be potential targets in repression.

There are two mechanisms of repression by Ssn6-Tup1

a) Targeting chromatin structure
    i. Direct interactions with the nucleosome,
    ii. Recruitment of chromatin modifying factors ,
b) Targeting RNA Pol II machinery.

3.2.1 Targeting Chromatin Structure

Chromatin is a dynamic structure composed of compacted nucleosome structures, which fold into higher-order structures. Chromatin exists in two forms: euchromatin (trascriptionally active) and heterochromatin (trascriptionally inactive). Chromatin shields DNA and forms a dais for harboring transcription regulators. It acts like a gate. Activators open the gate and repressors close the gate. The state of the nucleosmes determines the accessibility of DNA to replication, repair and transcription. Chromatin remodeling is the vital step in gene regulation.

3.2.1.1 Direct interactions with the nucleosome

Ssn6-Tup1 is known to interact with the amino termini of H3 and H4. The 121-385 amino acid domain of Tup1 has strongest interaction with H3 and H4. This domain is dominated with charged amino acids. The N-terminal domain (amino acids 21-28) of H3 that interacts with Tup1 has sites for acetylation and phosphorylation. Acetylation leads to gene activation and Tup1 causes repression which might suggest that there is a competition for the H3 amino

terminus between co-activator and co-repressor. The binding of Tup1 decreases with the acetylation of H3 and H4 (Edmondson DG, Genes Dev. 1996). Mutations in the amino termini of H3 and H4 severely disturb Tup1 association with target loci and lead to derepression (a-cell-specific genes, DNA damage response genes, etc) (Judith K. Davie *et al.*, Mol. Cell. Biol. 2002). *MAT a* cells express *STE6* gene. There is similarity in the positioning of nucleosomes in *MAT a* cells, which lack *MAT α2* repressor and tup1 deleted *MAT α* cells. Both mutations in histone H4 and deletion of Tup1 disrupt nucleosome positioning in *STE6*. This indicates that Tup1 represses transcription by the organization of a repressive chromatin structure (Cooper *et al*. 1994).

Ssn6-Tup1 complex is known to spread along chromatin domain of an *MAT a* cell-specific gene, *STE6,* which is roughly 4 kb, in repressed state. Four Tup1 molecules associate with a dinucleosome through deacetylated H3 and H4, and form a scaffold. This scaffold stabilizes the positions of the nucleosomes and organizes the chromatin in a repressive conformation. This indicates the architectural function of the complex (Ducker and Simpson 2000).

SUC2 gene has nucleosomes organized in UAS and TATA box which are the major targets of gene regulation. In repressed state nucleosomes shield the UAS and TATA box. The repressor (Mig1) binding site is the linker region of DNA between the two nucleosomes. Deletion of Tup1 and Ssn6 disrupts characteristic chromatin structure organization of the entire *SUC2* locus (Gavin and Simpson, 1997). Similar architecture of chromatin with protected regulatory DNA sequences and regulator binding nucleosome-free hypersensitive region is found in repressed state of *PHO5* (Svaren and Horz, 1997), *ADH2* (Verdone *et al.*, 1996) and in a-type cell-specific genes (Simpson *et al.*, 1993).

3.2.1.2 Recruitment of chromatin modifying factors

Chromatin folding is quite dynamic, and the degree of folding directly influences the activity of DNA in transcription, replication, repair and recombination. Nucleosomes undergo acetylation-deacetylation, phosphorylation-dephosphorylation, methylation-demethylation and ubiquition-deubiquition which trigger the conversion of euchromatin to heterochromatin. Histone acetylation and deacetylation majorly regulate repression of eukaryotic promoters. Tup1-Ssn6 is known to associate with histone deacetylases.

Histone deacetylases (HDACs) are classified into class I consisting of Rpd3, Hos1 and Hos2 and class II consisting of Hda1 (Grozinger, C.M., Hassig, C.A., and Schreiber, S.L. 1999. *Proc. Natl. Acad. Sci.* 96: 4868–4873.). The acetylation microarrays unveiled the division of labor of yeast HDAC (Robyr *et al*, Cell 2002). Rpd3 and Hda1 are two principal HDACs playing a vital role in transcription repression by deacetylating distinct promoters and gene classes where they are recruited by co-repressors. Disruption of Class I HDAC antagonizes Ssn6–Tup1 repression and lead to hyperacetylation of H3 and H4. Rpd3 and Hos2 are known to co-immunoprecipitate with Ssn6-Tup1.

Rpd3 is known to associate with several subunits like Sin3, Sap30, Sde3 and Pho23 to form Rpd3 complex (Kasten MM, Mol. Cell. Biol. 1997). Rpd3 is known to strongly deacetylate all four core histone proteins adjacent to the URS elements at INO1 (Suka N *et al*., Molecular Cell 2001). Rpd3 deacetylates the ORF of ENA1 (Wu J, Mol. Cell 2001). Rpd3 has been evolutionarily conserved. It is also known to associate with Sin3. Rpd3 deletion upregulates gene expression of 170 genes. Rpd3 forms two functionally different complexes, Rpd3L (large) and Rpd3S (small). Rpd3L is transcriptional regulator, recruited by sequence-specific DNA-binding proteins such as Ume6 to promoters, to represses gene expression by deacetylating proximal histones. Rpd3S recompacts chromatin after transcription of certain genes. It is known to regulate late replication timing (Knott SR, Genes Dev. 2009). Rpd3 has transcriptional activation properties at the telomeres indirectly by repressing histone genes. Its deletion is known to down regulate 264 genes and most of them are telomeric genes (Bernstein BE, PNAS 2000).

Hda1 of budding yeast is related to mammalian class II HDAC. Hda1 deacetylates subtelomeric domains containing normally repressed genes that are required for gluconeogenesis, growth on carbon sources other adverse growth conditions. Hda1 associates with two non catalytic subunits Hda2 and Hda3 to form Hda1 complex (Wu J, PNAS 2001). Through Ssn6-Tup1, Hda1 complex is known to be recruited to the promoters and modifies H2B and H3. Had 1 is also known to deacetylate more than 69% of the intergenic regions in a Tup1-independent manner (Robyr Y *et al*, Cell 2002). The subunits Hda2 and Hda3 may serve as unspecific DNA binding motifs and help Hda1 get into proximity with H2B and H3 (Lee JH, J Mol. Biol. 2009). Hos1/Hos3 and Hos2 preferentially affect ribosomal DNA and ribosomal protein genes, respectively.

Hyperacetylation by histone acetyltransferases (HATs) prevents Ssn6-Tup1 binding to chromatin. During repression the repressors call for Ssn6-Tup1 and this complex thereby recruits HDAC and leads to hypoacetylation. Acetylated lysine residues of nucleosomal histones results in allosteric changes in nucleosomal conformation, destabilization of internucleosomal contact and increases accessibility of DNA to transcription factors. HATs are classified into three families: GNAT (Gcn5-related N-acetyltransferases), MYST (MOZ, Ybf2, Sas2 and Tip60) and p300/CBP. HATs associate with transcriptional co-activators and cause gene activation.
The action of both HATs and HDACs maintains histone acetylation in constant flux. Ssn6-Tup1 alters the flux towards deacetylation of H3 and H4 by recruiting HDACs. The interactions between Ssn6-Tup1 and underacetylated H3 and H4 might sterically hinder HATs and stabilize repressed state.

The repression domain (Tzamarias and Struhl, Nature 1994) and the histone binding domain (Edmonson *et al*., Genes Dev. 1996) of Tup1 overlap. Recruitment of HDAC leads to deacetylation and this might enhance the histone binding ability of Ssn6-Tup1 to the nucleosomes. This might suggest that these two functions are tightly coordinated and interdependent.

3.2.2 Targeting RNA Pol II machinery

Ssn6-Tup1 complex is known to repress genes even in the absence of nucleosomes, *in vitro*. α2 mediated repression involves Ssn6-Tup1 (Szeto L and Broach JR, Mol Cell Biol. 1997). *In vitro* experiments are performed using DNA templates without histones. Transcription inhibition studies are performed using template with *α2* repressor and *MCM1* binding sites upstream of *CYC1* promoter which has no activator binding site and a control template without *α2* repressor and *MCM1* binding sites. A four-fold reduction in basal transcription is observed by α2 repressor. This suggests that *α2* repressor, in turn the co-repressor complex awks transcriptional machinery (Herschbach, Nature 1994).

RNA polymerase II holoenzyme consists of RNA polymerase II, the general transcription factors (GTF) TFIIB, TFIIF, and TFIIH and the Srb (Suppressors of RNA polymerase B) proteins Srb 2, Srb 4 to Srb 11 (Hengartner C, Genes Dev 1995). The Srb10 and Srb11 proteins form a kinase-cyclin pair in the RNA polymerase II holoenzyme and are isolated as suppressors of mutations in RNA polymerase II carboxy-terminal domain (CTD). CTD is unphosphorylated

during transcription initiation and is phosphorylated during transcription elongation. In the srb10 mutant holoenzyme, CTD is 10 fold less phosphorylated *in vitro*. (Liao, S, Nature 1995).

Ssn6-Tup1 complex mediates glucose repression of *SUC2* transcription through Mig1. Mig1 deletion partially relieves repression of *SUC2* genes. This implies that Ssn6-Tup1 has a Mig1-independent repression mechanism. Snf1 is an activator of glucose responsible genes, which is known to relieve transcriptional repression by Ssn6-Tup1. Deletion of Ssn6 or Tupl allows *SUC2* expression in a Snfl deletion mutant, indicating that the Snfl can release repression by the Ssn6-Tup1 proteins. There might be other proteins that mediate effect of Tup1-Ssn6 on *SUC2* expression. Snf1 does not phosphorylate Ssn6 (Schlutz *et al*., Mol. Cell. Biol. 1990). Other genes, Ssn2, *etc*. are also identified to be suppressors of Snf1. Mutations in these genes have synergy with *mig1* mutations, to suppress *snf1* deletion. Ssn3 and Ssn8 are Srb10 and Srb11 respectively, which are components of RNA Polymerase II mediator complex. Ssn6-Tup1 might have interactions with the mediator complex and thereby repress *SUC2* expression even in the absence of Mig1. (Vallier LG and Carlson M, Genetics 1994). If there are two parallel pathways in repression, then both pathways require Ssn6-Tup1 since deletion of Ssn6-Tup1 causes a complete derepression,.

The kinase subunit Are1 is known to impair α2 repression of a-specific genes in *MAT α* cells. Are1 is identical to Srb10. (Wahi M, and A. D. Johnson, Genetics 1995). *SUC2* glucose repression and α2 repression depend on the Ssn6-Tup1 complex and Srb10-Srb11 suggesting that Ssn6-Tupl mediates repression targeting the general transcription machinery.

Genetic evidence implicates the Srb10-11 complex in control of a diverse set of genes, which include those involved in glucose metabolism, meiosis, phosphate repression of acid phosphatase and α2 repression of a-specific genes (Kuchin *et al*., PNAS 1995). The repression defects caused by srb10 and srb11 mutations are modest in natural promoters regulated by Ssn6-Tup1: cell type (*MFA1*, *MFA2*), glucose (*SUC2*), oxygen (*ANB1*), and DNA damage (*RNR2*). (Lee M *et al*., Genetics 2000). Srb10-Srb11 had very modest role in the repression of natural promoter *CYC1* and synthetic promoter with *LEU2* UAS and *HIS3* TATA (Kuchin S and Carlson M, Molecular and Cellular Biology, 1998).

CTDK-I is a CTD kinase protein comprised of Ctk1and Ctk2 subunits. Ctk1 and Ctk2 are similar to Srb10-Srb11. Like *srb10* mutation, mutation of *ctk1* causes mild defects in glucose repression of *SUC2* and *GAL1*. Mutated *ctk1* acts synergistically with *mig1* mutations to relieve repression. Ctk1 and Srb10 are functionally independent as, overexpression of Srb10 does not suppress defects caused by the Ctk1 deletion. Ctk1 does not interact with the Srb11 cyclin and Srb10 does not interact with the Ctk2 cyclin. Srb10-Srb11 and CTDK-I contribute to repression by Ssn6-Tup1. (Kuchin S and Carlson M, Mol. Cell. Biol, 1998).

CTDK-I stimulates the elongation efficiency of RNA polymerase II (Lee JM, Greenleaf AL, J. Biol. Chem 1997). It is known that Gal4 is phosphorylated during activation. CTD and its associated kinase-cyclin pairs (Srb10-Srb11 and CTDK1) could be involved in the phosphorylation of activators which is hindered in the presence of Ssn6-Tup1. Ssn6 and Tup1 are known to be phosphorylated *in vivo*. (Redd M *et al*., J. Biol. Chem 1997). Phosphorylation of Ssn6- by the above two kinase-cyclin pairs could play a role in transcription regulation. Ssn6-Tup1 could stimulate Srb10-Srb11 kinase-cyclin pair to phosphorylate a GTF or Ssn6-Tup1 tightly binds to the kinase-cyclin pairs thereby preventing transcription.

3.2.3 Localization in the genome

A statistical genome-wide analysis discloses that Hda1 represses 30 % of Tup1- repressed genes and Srb10 represses only 15% of Tup1- repressed genes. When *HDA1* is deleted there is hypoacetylation observed at certain promoters, yet repressed. In srb10 and hda1 double mutant strain only 50% of the Tup1-repressed genes are repressed. Thus Tup1-mediated repression occurs by multiple, partially overlapping mechanisms, some of them are not identified yet. Taking this into consideration that *HDA1* deletion affects subtelomeric genes (Robyr *et al*, Cell 2002), microarray analysis revealed that 30% of Tup1 repressed genes are subtelomeric. The transcriptional functions of Hda1 and Srb10 in subtelomeric regions are more dependent on Tup1-mediated repression than they are at internal chromosome positions.

3.2.4 Evolutionary conservation of Tup1 and Ssn6

Tup1 of *Saccharomyces cerevisiae* shares similarity of structure and function with *Schizosaccharomyces pombe* Tup1 (Mukai *et al*., Mol. Cell. Biol. 1999) and Groucho of

Drosophila (Guoqing *et al*., Genes Dev. 1999). Mammalian TLE (transducin-like Enhancer) proteins have sequence similarity with Tup1 and Groucho (Palaparti *et al*., J. Biol. Chem. 1997). TLE proteins are known to interact with yeast Ssn6 and mammalian Ssn6-like proteins. Ubiquitously transcribed tetratricopeptides are on the Y or X chromosome (UTY/X) in mammalian cells and exhibit similar size and structure like Ssn6. Ssn6 interacts with TLE proteins and results in transcriptional repression in mammalian cells (Grbavec *et al*., Biochem. J. 1999). Ssn6-Tup1-like corepressors interact with chromatin and modify histone post-translationally. Their structure and function is conserved across evolution, underscoring the importance of these functions to the regulation of gene expression.

3.3 Silencing

Chromatin profiling occurs both at local and global level. Whether it is local or global, inhibition of gene expression happens at the level of nucleosomes. Repression by Ssn6-Tup1 is at the local level, and is promoter-specific. At global level, promoter-independent gene expression is inhibited by silencing. Silencing is a phenomenon that leads to formation of a specialized chromatin structure called silenced chromatin or heterochromatin that blocks expression of most genes. Heterochromatin is stable and is inherited over many generations. *Saccharomyces cerevisiae* harbors three classes of genetic loci which undergo silencing; ribosomal DNA repeats, telomeres and mating type loci. The silent chromatin in these loci is comparable to the heterochromatin of higher eukaryotes. Yeast heterochromatin being inaccessible to DNA modifying agents, replicating in late S-phase, and reduced acetylation of H3 and H4 N-terminal lysine residues show a signature of metazoan heterochromatin (Bi X, Broach JR, Mol. Cell. Biol. 1997).

3.3.1 Silenced terrains of the genome

Silencing in budding yeast is restricted to two homothallic mating type loci (HML and HMR), telomeres and r-RNA encoding DNA. The HML and HMR are on Chromosome III. The silencers *HMR-E* and *HMR-I* flank *HMR* and the silencers *HML-E* and *HML-I* flank *HML*. E-silencers are essential. At HML both E (centromere-distal) and I (centromere proximal) silencers

can promote silencing independently (Mahoney DJ and Broach JR, Mol. Cell. Biol 1989). In case of HMR E (centromere proximal) is the stronger silencer than I silencer (centromere distal) (Brand AH *et al*., Cell 1985). The silencers are genetically dissected into smaller functional elements.

SILENCER	CONSENSUS SEQUENCES
HML - E	Rap 1 binding site, ARS consensus sequence
HML - I	Rap 1 binding site, ARS consensus sequence, Abf1 binding site
HMR - E	Rap 1 binding site, ARS consensus sequence, Abf1 binding site
HMR - I	ARS consensus sequence, Abf1 binding site

Table 2: Consensus sequences in HM silencers (Bi X *et al*., PNAS, 1999).

Silencers function in recruiting Sir (Silent Information Regulator) complex. HMR-E and HML-I have binding site for ORC, Rap1, and Abf1. Rap1 and Abf1 are two of the most common transcription activators in *S. cerevisiae*. ORC is the origin recognition complex for initiation of DNA replication. Mutations in two of the three sites result in loss of silencing. Juxtaposition of three proteins may provide a site for Sir protein binding. HMR-I has a supporting role but does not recruit Sir proteins due to the absence of Rap1 binding sites. Interactions of Sir protein complex with Rap1 play a key role in the assembly of Sir complex. Long-range cooperativity is observed between the silencers in establishing silenced chromatin. Single sequence of Rap1 binding site, ARS consensus sequence or Abf1 binding site can augment silencing cooperating with a distant silencer (Boscheron C, The EMBO J 1996). Telomeres are the ends of the chromosome and have reduced rates of recombination. They are inaccessible to DNAases and DNA modifying enzymes which is attributed to the compact chromatin structure. Telomeric silencers have an array of multiple Rap1 binding sites of $(TG_{1-3})_n$ repeats which recruit Sir complex and lead to the formation of silenced chromatin (Gilson E, Roberge M, *et al*. J. Mol. Biol, 1993).

3.3.2 Silencing Machinery

Formation of heterochromatin requires cis-acting and trans-acting elements. The cis-acting elements are silencers, regulatory sequences to which the trans-acting factors come and bind. They act as nucleation sites for recruiting factors which are proteins that regulate

heterochromatin formation. In *Saccharomyces cerevisiae* Abf1, Rap1, Orc2 and Orc5 are recruited to the silencers. These further recruit Sir proteins, which thereby lead to the formation of silent chromatin. Sir2, Sir3 and Sir4 interact with each other and also with the nucleosomes to establish and maintain heterochromatin (Hoppe GJ, Tanny JC, *et al*. Mol. Cell. Biol, 2002).

3.3.2.1 Sir2

Sir2 is the catalytic unit of the Sir complex. It belongs to Sirtuin family and functions as a NAD^+ dependent histone deacetylase. One molecule of NAD^+ is cleaved into ADP-ribose and nicotinamide per acetyl group removed. The acetyl group is transferred to ADP-ribose, forming the product 2'-*O*-acetyl-ADPribose, which equilibrates to a combination of 2'-*O*-acetyl-ADP ribose and 3'-*O*-acetyl-ADP ribose (Jackson MD and Denu JM, *J. Biol. Chem* 2002). The energy produced during this reaction might be utilized in conformational changes of DNA and nucleosomes to form silenced chromatin. *O*-acetyl-ADP ribose enhances the association of Sir3 with Sir2/Sir4 in multitude and spurs structural rearrangement in the SIR complex. Sir2 activity modulates the assembly of the SIR complex through both histone deacetylation and *O*-acetyl-ADP ribose synthesis (Liou GG, *et al*., Cell, 2005). On the other hand, Sir3 chimera-bearing Hos3, a NAD(+)-independent histone deacetylase, replaces for Sir2 in silencing. Sir3-Hos3 HM loci in strains lacking all five *O*-acetyl-ADP ribose -producing deacetylases, indicating that *O*-acetyl-ADP ribose is not necessary for silencing (Chou CC, *et al*., Mol. Cell, 2008).The H3 and H4 histone tails are hypoacetylated, and the DNA is generally refractory to modifying enzymes. Sir2 deacetylates a critical acetyl mark on histone H4K16. Multiple lysine residues within the N-terminal tails of H3 (K9 and K14) and H4 (K5, K8, K12, and K56) can be deacetylated by Sir2, but the influence of individual residues on the formation and stability of silent chromatin varies (Borra MT *et al*, Biochemistry 2004). It renders large regions of chromosome transcriptionally inactive in a promoter-independent manner like in higher eukaryotes. The major, evolutionarily conserved, histone acetyltransferase that modifies substrates for Sir2 by targeting H4 K16 in cells is Sas2p (Kimura A, Nat Genet 2002).

3.3.2.2 Sir3

Sir3 has no known enzymatic activity but has structural role in the assembly of silent chromatin. Sir3 binds to mononucleosomes, nucleosome array and DNA. Sir3 might crosslink

individual nucleosomal arrays into supramolecular structures and mediate global reorganization of chromatin (George PT *et al*, PNAS 2001). The H4 N-terminal deletion causes unfolding of chromatin or some sliding of ordered nucleosomes and derepresses HM loci. H4 plays major role in silencing (Kayne PS, Cell 1988). Single amino acid mutations K16Q, R17G, H18G, I24P and G28P of H4 disrupted the interactions between H4 and Sir3. Sir4, RAP1 and histones co-immunoprecipitates with Sir3 (Hetch A, Nature 1996). Sir3 and Sir4 form heterodimers. The C-terminal region of Sir3 (832-978 amino acids) mediates Sir3 dimerization. One half of Sir3 dimer (464-728 amino acids) interacts with Sir4 dimer to form a stable complex. The Hill coefficient of Sir3 (monomer and dimer) binding to Sir4 is greater than 3. Dimerization of Sir3 contributes to strong affinity of Sir4 (King DA, *et al*., J. Biol. Chem. 2006). Sir3 binds to Rap1 *in vitro* in the absence of other Sir proteins. Sir3 and Sir4 can bind to Rap1 bind *in vivo*. Mutations in Rap1 C-terminus that reduce Sir3 binding cause silencing defects at HM loci and telomeres (Moretti P *et al*., Genes Dev. 1994). The cooperative assembly of Sir4 dimer in complex with Sir3 dimer and their interactions with Rap1 and deacetylated histone tails promotes the spreading of Sir protein complexes to establish transcriptionally silent domains of DNA. Deacetylation of histone H4K16 is critical for the binding of Sir3 and Sir4 to histone H4 peptides in vitro (Liou GG, *et al*., Cell, 2005). Sir3 dictates the specificity of direct and functional interactions between the SIR complex and chromatin. The association of Sir3 with chromatin is disrupted by H4K16 acetylation or mutation of histone H4K16 to H4A16 residues that are implicated in assembly of silent chromatin (Johnson A, *et al*., Mol. Cell, 2009).

3.3.2.3 Sir4

Sir4 has a structural role in silencing. It bridges Sir2 (catalytic component of Sir complex) and Sir3 (histone and DNA associating component of Sir complex) and helps in the propagation of silent chromatin. Sir4 binds the N-terminal domain of Sir2 spanning amino acids 94–198 and 422–562 (Cockell MM *et al*, Genetics 2000). Sir3 and Sir4 interact with H3 and H4 N-termini, which serve as chromosomal anchoring sites for Sir3 and Sir4 (Hecht A, *et al*, Cell, 1995). Deacetylation of histone H4K16 is critical for the binding of Sir3 and Sir4 to histone H4 peptides in vitro. *O*-acetyl-ADP ribose increases the association of Sir3 with Sir4 (Carmen AA *et al*, J. Biol. Chem. 2002; Liou GG, *et al*., Cell, 2005). Disruption of *SIR4* gene prevents the aggregation and perinuclear positioning of telomeres. SIR4 may provide the means by which

telomeric heterochromatin is tethered to the nuclear periphery (Hecht A, *et al*, Cell, 1995). Sir4 helps plasmids divide equally at mitosis (Ansari A and Gartenberg MR, Mol. Cell. Biol 1997). It interacts with Dis1p/Ris1p required for recombination during mating type switching (Zhang Z, Buchman AR. 1997. Mol. Cell. Biol). Sir4 interacts with ubiquitin hydrolases, Dot4 and Ubp3 (Kahana A, Gottschling DE, Mol.Cell. Biol 1999).

3.3.2.4 Sir1

Sir1 has a unique role in the establishment of silencing at HML. Sir1 mutant strains had different populations. Majority of cells has derepressed HML locus (Pillus L, Rine J, *Cell* 1989). Sir1 can bind directly to ORC1, the largest of the ORC subunits, and that targeting of Sir1 to Orc1 at a silencer is to establish a silenced state (Triolo T, Sternglanz R, Nature 1996). Sir1 interacts with Sir4. Sir1 has a role in the establishment but not the maintenance of repression of silent mating type genes, whereas Sir2, Sir3, and Sir4 are required for maintenance. Targeting Sir1 alone to HM loci establishes silencing. Recruiting Sir1 to telomeres stabilizes silencing at telomeres (Chien CT, *et al*, Cell, 1993). When Sir3 and Sir4 are sequestered to HMR-E silencers, silencing is restored (Marcand S, *et al*, Genes Dev, 1996).

3.3.3 Nucleation and Spreading of Silent chromatin

Protein- protein interactions are the major premise for the establishment of Silent chromatin. Sir1 is recruited to the silencer and interacts with Orc1 subunit of ORC complex. Rap1 binds to the silencers. Interactions of Sir4 with Rap1 and Sir1 aid its recruitment to the silencer. Sir4 procures Sir2 as Sir2-Sir4 complex. Sir3 is known to interact with Rap1, Sir4 and perhaps Abf1. Sir3 interacts with histones H3 and H4 and Sir3 has more affinity for hypoacetylated histones. Sir2 procured with Sir4 has the catalytic activity and deacetylates the histones. This increases affinity of the Sir complex to chromatin. At telomeres Sir1 has no role and recruitment of Sir3 and Sir4-Sir2 complex is through Rap1 interactions.

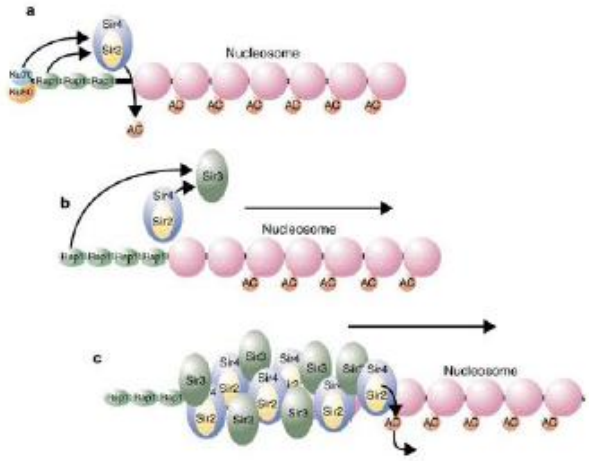


Figure 1: Spreading of SIR complex. (Adapted from Hild M and Paro R, Nat Cell Biol 2003).

Once nucleation complex (Rap1, Sir proteins, H3 and H4) has assembled, Sir2 and Sir3 contribute majorly for the Sir complex to spread stably both at telomeres and HM loci. The interaction between C-terminus of Sir3 and N-terminus of H4 is crucial for the stable formation of this complex. Acetylation of H4 lysines reduces association (Ka) of H4 to Sir3. The fully acetylated peptide has approximately 50-fold lower binding (Ka) relative to unacetylated peptide (Carmen AA *et al*, J. Biol. Chem. 2002). The sequential deacetylation of histones by Sir2 creates high affinity sites for Sir3 and Sir4. The subsequent deacetylation procures Sir3 and Sir4. Sir4 is recruited as a complex with Sir2. Now the newly loaded Sir2 deacetylates the acetylated H3 and H4 histone tails on the next nucleosome. The consecutive process of deacetylation of adjacent nucleosomes and loading additional Sir proteins allows the spreading of heterochromatin.

Silenced chromatin is permissive to certain binding proteins like enzymes that mediate homologous recombination, site-specific recombination (Cheng TH *et al*., PNAS 1998), and retrotransposon integration (Zou S *et al*. Genes Dev. 1996). Sir complex generated heterochromatin of HSP82 allows the constitutive binding of an activator, Hsf, and two components of the pre-initiation complex (PIC), TBP and Pol II. The silent chromatin at *HMR*a1 promoter efficiently recruits TBP and Pol II. These observations suggest that yeast heterochromatin has a mechanism that is different from steric interference and restricting factor

access to DNA but rather blocks a step subsequent to activator and PIC recruitment (Sekinger EA and Gross DS, Cell, 2001). On the contrary, Chen L and Widom J (Cell, 2005), conclude that silencing acts downstream of gene activator protein binding to strongly reduce the occupancy of TFIIB, RNA polymerase II, and TFIIE at the silenced promoters.

3.3.4 Boundary elements

The silent chromatin in yeast is only pertained to certain regions of the genome. The propagation of silent chromatin beyond these regions is stopped by boundary elements. They are DNA sequences that recruit barrier proteins to inhibit the spread of silent chromatin into actively transcribing region. Introducing sequences that do not favor nucleosome formation or sites (Rap1 binding sites) that aid nucleosome exclusion is one of the mechanisms for the establishment of boundaries of silent chromatin domains (Bi X *et al*, Mol. Cell. Biol. 2004). Subtelomeric anti-silencing regions (STAR) have Tbf1 or Reb1 binding sites which counteract silencer driven gene inhibition (Fourel G *et al*., EMBO Rep 2001). The boundary element of HMR contained a Ty1 LTR, and a tRNA gene (Donze D *et al*., Genes Dev 1999). Deletion of barrier elements would result in expansion of silent chromatin domain (West AG, Genes Dev. 2002). Directionality of the silencers also defines boundaries (Bi X and Broach R, Curr. Opin. Genet. Dev. 2001). A gradient of chromatin modifications, such as differing degrees of histone hyperacetylation or hypoacetylation on opposing sides of the resulting boundary element also define a boundary (Grewal SI, and Moazed D, Science 2003). Genetic and biochemical evidence show that Rpd3 antagonizes Sir-dependent repression of heterochromatin-adjacent regions. Rpd3 deacetylates H4K5. Mutations in H4K5, in *rpd3* deleted strain, has reduced binding of Sir2. This suggests that acetylated H4K5 has affinity for Sir2, and its deacetylation by Rpd3 antagonizes silencing. (Zhou J *et al*., Nucleic Acid Res 2009). Rpd3 might counteract Sir2 at the telomeres indirectly by repressing histone genes (Bernstein BE, PNAS 2000).

3.3.5 Position variegation Effect

S. cerevisiae, telomeres exert a position effect on the expression of genes located nearby. Position effect is a phenomenon where the expression of gene is effected by its location in the genome. When *URA3, TRPl, HIS3*, or *ADE2* is located near a telomere, the gene's transcription is repressed. The repression of above genes is reversible and both repressed and active

transcriptional states were inherited mitotically in a semi-stable manner. Conversion of repressed to active state and vice-versa appears to be under epigenetic control. Transcription of *URA3* is not repressed at a locus -20 kb away from the telomere, even though the 81 bp tract of $(TG_{1-3})_n$ Rap1 binding sequence was located adjacent to the gene. The telomeric position effect (TPE) appears to be a result of proximity to the end of the chromosome and is not simply due to the telomeric DNA sequence $(TG_{1-3})_n$ (Gottschling DE, Cell 1990).TPE can be considered as a gradient of transcriptional silencing along the chromosome limited by the SIR protein complex. When a *URA 3* gene is placed at different distance from the telomere, with increasing distance, away from the telomere, silencing reduced (Figure 2). Overexpression of Sir3 enhances position effect of telomeric genes (Renauld H, *et al,* Genes Dev, 1993). Telomere looping may also play a critical role in maintaining TPE. When Switching of telomeric states from repressed to actively transcribed states might eliminate subtelomeric Rap1 interactions. Loss of these interactions might disrupt core heterochromatin and Rap1p-mediated telomere looping (de Bruin D *et al*., Mol. Cell. Biol. 2000). Perinuclear localization also helps in establishing silent chromatin as the telomeres are located at the nuclear periphery and that the concentrations of Sir proteins are greater at the nuclear periphery (Andrulis ED *et al*., Nature 1998). The rate of Sir protein assembly and spreading also depends on its location. Assembly of Sir complex is rapid at HMR locus than at the telomere VI-R. The recruitment of Sir proteins is similar at HMR-E and telomeric silencers but the rate of spreading varies. Insertion of HMR-E adjacent to the telomere VI-R increased the rate of Sir2p association with the telomere (Lynch PJ and Rusche LN, Mol. Cell. Biol. 2009).

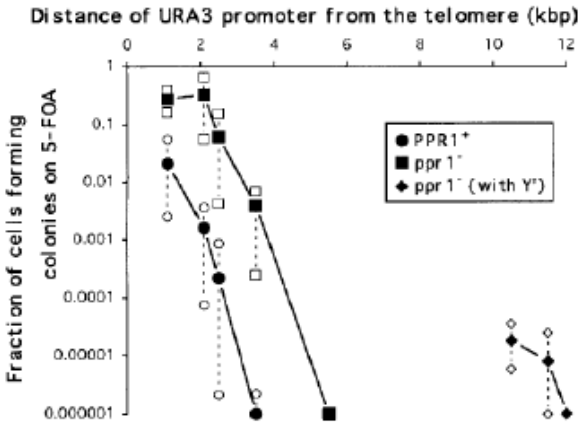


Figure 2: Distance dependence of telomeric silencing (Adapted from Renauld H, *et al,* Genes Dev, 1993)

3.3.6 Bridge between Repressors and Silent Information Regulators

Ssn6-Tup1 mediated repression attacks the acetylated histone tails by recruiting HDACs and binds to the hypoacetylated promoter and spreads to a limited extent. Sir complex also attacks the acetylated histone tails and spread to several kilo bases along the chromosome in a promoter-independent fashion. Ssn6-Tup1 seems to share similarities to Sir protein–mediated silencing mechanism. Sum1 is a repressor that regulates meiotic gene expression leading to their repression in mitotic cells. It is promoter-specific (Xie JX, EMBO J. 1999). A dominant mutant of Sum1 called Sum1-1 restores silencing at HM locus in mutant strains lacking Sir2, Sir3 and Sir4 (Klar AJ *et al*. Genetics, 1985; Laureson P and Rine J, Genetics, 1991). Sum1-1 associates with silencer and with the help of deacetylase Hst1 propagates silent chromatin (Sutton A *et al*., Mol. Cell. Biol, 2001). When *HST1* is deleted, Sum1-1 associated with *HMR-E* silencer but did not spread. Even in the case where *RFM1*, bridging protein between Sum1-1 and Hst1, is deleted, Sum1-1 did not spread. Sum1-1 associates with *HMR-E* silencer and Rfm1. Rfm1 in turn recruits Hst1 which deacetylates histones and helps Sum1-1, Rfm1 and Hst1 complex propagate to form heterochromatin. H4 requirement for Sum1-1 to spread imply the stronger affinity of Sum1-1 with H4 over Sum1(Lynch PJ, *et al*, Mol. Cell. Biol., 2005). The propagation of silent chromatin by Sum1-1 is similar to Sir-mediated silencing. Sum1-1 is also known to circumvent the necessity of Sir proteins in silencing HM loci and increase inhibition at telomeres (Chi, M. and Shore, D., Mol. Cell. Biol., 1996). A single critical amino acid has converted a

promoter-specific repressor to a silencing protein (Rusche LN and Rine J, Genes Dev. 2001) underscoring the narrow margin between repression and silencing and accentuates the similarities in their mechanisms.

3.4 Inhibition kinetics

3.4.1 Michaelis-Menten equation

The basic enzyme substrate reaction resulting in a product formation is

$$E + A \underset{k2}{\overset{k1}{\rightleftharpoons}} EA \xrightarrow{k3} E + P$$

Formation of enzyme substrate (EA) complex is a reversible process but the formation of product (P) is an irreversible step. Rate of enzyme catalyzed reactions increase with increase in substrate concentration but becomes saturated at very high substrate concentrations. It is assumed that the steady state is reached shortly after the beginning of the reaction. The enzyme substrate concentration changes much slower than concentrations of product and substrate.

The focus on enzyme catalyzed reaction begins with the fact that initial rate of reaction is directly proportional to the formation of product with time

$$v0 = \frac{d[P]}{dt} = k3\ [EA] \qquad \text{Eq: 1}$$

The rate of enzyme substrate complex formation is

$$\frac{d[EA]}{dt} = k1\ ([E][A]) - (k2 + k3)[EA] = 0 \qquad \text{Eq: 2}$$

The total enzyme concentration is considered constant and is equivalent to free enzyme and enzyme bound to substrate.

$$[E]t = [E] + [EA] \qquad \text{Eq: 3}$$

Substituting Eq: 3 in Eq: 2

$$k1[A]\ ([E]t - [EA]) - (k2 + k3)[EA] = 0 \qquad \text{Eq: 4}$$

$$k1[A][E]t = k1[A][EA] + (k2 + k3)[EA] \qquad \text{Eq: 5}$$

$$[A][E]t = [A][EA] + [EA]\frac{(k2+k3)}{k1} \qquad \text{Eq: 6}$$

The apparent dissociation constant of enzyme substrate complex is KA

$$KA = (k2 + k3)/k1 \quad \text{Eq: 7}$$

Substituting KA in Eq: 6

$$[A][E]t = (KA + [A])[EA] \quad \text{Eq: 8}$$

$$v0 = \frac{d[p]}{dt} = k3\ [EA] = k3\ [A][E]t/(KA + [A]) \quad \text{Eq: 9}$$

$$Vmax = k3[E]t \quad \text{Eq:10}$$

$$v0 = Vmax\,[A]/(KA + [A]) \quad \text{Eq:11}$$

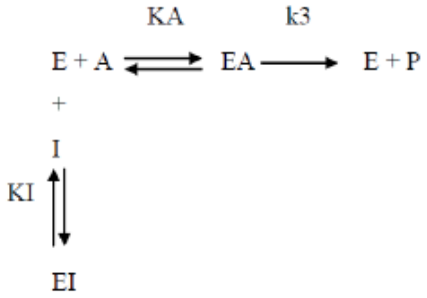
Equation 11 is the fundamental Michaelis–Menten equation, the constant value KA as the Michaelis constant and the constant value Vmax as the maximal velocity of reaction.

3.4.2 Inhibitors and enzyme inhibition

Inhibitors bind to enzyme or enzyme substrate complex and reduce enzyme catalysis. There are two types of inhibition: reversible and irreversible inhibition. In irreversible inhibition the inhibitor forms a irreversible covalent bond with the enzyme and terminates the activity of the enzyme. Reversible inhibitors usually form non-covalent complexes with enzyme thereby lowering the amount of enzyme available for the reaction. Depending on the influence on the enzyme kinetics inhibitors are divided into three categories: i) competitive inhibition, ii) uncompetitive inhibition and iii) non- competitive inhibition.

3.4.2.1 Competitive inhibition

The competitive inhibitor competes for the same binding site on the enzyme as that of the substrate. Most competitive inhibitors are structural analogues of the substrate. The inhibtor forma an abortive and non-productive comple with the enzyme.



Inhibitor associates with some amount of the enzyme.

$$[E]t = [E] + [EA] + [E][I] \qquad \text{Eq:12}$$

The dissociation constant of enzyme inhibitor complex is KI

$$KI = [\mathrm{E}][\mathrm{I}]/[\mathrm{EI}] \qquad \text{Eq:13}$$

The dissociation constant of enzyme substrate complex is KA

$$KA = [\mathrm{E}][\mathrm{A}]/[\mathrm{EA}] \qquad \text{Eq:14}$$

Substituting Eq:13 and Eq:14 in Eq: 12 gives

$$[E]t = [E]\left(1 + \frac{[A]}{KA} + \frac{[I]}{KI}\right) \qquad \text{Eq:15}$$

Substitue Eq: 15 in Eq: 14and rearrange

$$[EA] = \frac{[A]}{KA}\left\{\frac{[E]t}{1 + \frac{[A]}{KA} + \frac{[I]}{KI}}\right\}$$

$$[EA] = \frac{[E]t\,[A]}{KA\left(1 + \frac{[I]}{KI}\right) + [A]} \qquad \text{Eq:16}$$

It is known that

$$v0 = \frac{d[P]}{dt} = k3\,[EA] \qquad \text{Eq: 1}$$

Substituting Eq: 16 in Eq: 1 gives

$$v0 = k3\,\frac{[E]t\,[A]}{KA\left(1 + \frac{[I]}{KI}\right) + [A]}$$

$$v0 = \frac{Vmax\,[A]}{KA\left(1+\frac{[I]}{KI}\right)+[A]} \qquad \text{Eq:17}$$

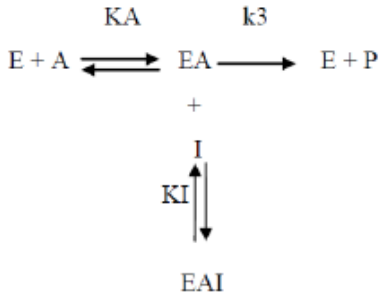
The apparent dissociation constant of the substrate in a competitive inhibition reaction is

$$KA\ app = KA\left(1+\frac{[I]}{KI}\right)$$

In a competitive inhibition reaction the dissociation constant of the substrate is increased by a factor of $\left(1+\frac{[I]}{KI}\right)$ but Vmax remains unchanged. The presence of the inhibitor can be overcome by higher substrate concentrations.

3.4.2.2 Uncompetitive inhibition

An uncompetitive inhibitor binds reversibly to the enzyme-substrate complex yielding an inactive enzyme-substrate-inhibitor (EAI) complex and does not resemble the substrate of the reaction it is inhibiting.. The inhibitor does not bind to the free enzyme.



It is known that

$$v0 = \frac{d[P]}{dt} = k3\ [EA] \qquad \text{Eq: 1}$$

The dissociation constant of enzyme substrate complex is KA

$$KA = [E][A]/[EA] \qquad \text{Eq:14}$$

The dissociation constant of the enzyme-substrate-inhibitor complex is KI

$$KI = [EA][I]/[EAI] \qquad \text{Eq:18}$$

Substitute Eq: 14 in Eq: 18

$$[EAI] = \frac{[EA][I]}{KI} = \frac{[E][A][I]}{KA*KI} \qquad \text{Eq:19}$$

Total enzyme concentration is

$$[E]t = [E] + [EA] + [EAI] \qquad \text{Eq:20}$$

Substitute Eq: 14 and Eq: 19 in Eq: 20

$$[E]t = [E](1 + \frac{[A]}{KA} + \frac{[A][I]}{KA*KI}) \qquad \text{Eq:21}$$

Substitue Eq: 21 in Eq: 14and rearrange

$$[EA] = \frac{[A]}{KA} \{ \frac{[E]t}{1+\frac{[A]}{KA}+\frac{[A][I]}{KA*KI}} \}$$

$$[EA] = \frac{[E]t\,[A]}{KA+[A]\left(1+\frac{[I]}{KI}\right)} \qquad \text{Eq:23}$$

It is known that

$$v0 = \frac{d[P]}{dt} = k3\ [EA] \qquad \text{Eq: 1}$$

Substituting Eq: 22 in Eq: 1 gives

$$v0 = k3\ \frac{[E]t\,[A]}{KA+[A]\left(1+\frac{[I]}{KI}\right)}$$

$$v0 = \frac{Vmax\,[A]}{KA+[A]\left(1+\frac{[I]}{KI}\right)} \qquad \text{Eq: 24}$$

The apparent dissociation constant of the substrate in a uncompetitive inhibition reaction is

$$KA\ app = \frac{KA}{\left(1+\frac{[I]}{KI}\right)}$$

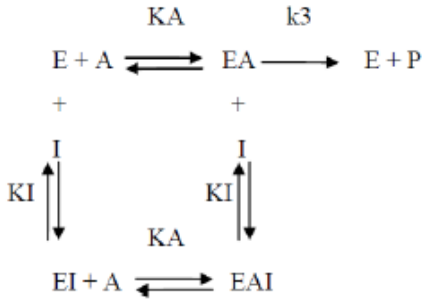
The apparent Vmax in a uncompetitive inhibition reaction is

$$Vmax\ app = \frac{Vmax}{\left(1+\frac{[I]}{KI}\right)}$$

In uncompetitive inhibition reaction both Vmax and the dissociation constant of the substrate is decreased by a factor of $\left(1+\frac{[I]}{KI}\right)$. Uncompetitive inhibition is effective when substrate concentration is high. Decreasing the substrate concentration might relieve the inhibition.

3.4.2.3 Non-competitive inhibition

In this type of inhibition the inhibitor can bind to free enzyme or enzyme-substrate complex. It binds to the allosteric enzyme at a site where substrate does not bind. This binding might induce structural changes in the enzyme which in turn reduces the binding of substrate to the enzyme. The effective concentration of active enzyme is reduced and thus affects enzyme catalysis.



The dissociation constant of the substrate is

$$KA = \frac{[E][A]}{[EA]} = \frac{[EI][A]}{[EAI]} \qquad \text{Eq:25}$$

The dissociation constant of the inhibitor is

$$KI = \frac{[E][I]}{[EI]} = \frac{[EA][I]}{[EAI]} \qquad \text{Eq:26}$$

Substituting Eq: 26 in Eq: 25

$$[EAI] = \frac{[E][A][I]}{KA * KI} \qquad \text{Eq:27}$$

Total enzyme concentration is

$$[E]t = [E] + [EA] + [EAI] + [EI]$$

$$[E]t = [E]\left(1 + \frac{[A]}{KA} + \frac{[A][I]}{KA * KI} + \frac{[I]}{KI}\right) \qquad \text{Eq:28}$$

Plug Eq: 28 in Eq: 25

$$[EA] = \frac{[A]}{KA} \frac{[E]t}{\left(1 + \frac{[A]}{KA} + \frac{[A][I]}{KA * KI} + \frac{[I]}{KI}\right)}$$

$$[EA] = \frac{[E]t\,[A]}{(KA + [A])\left(1 + \frac{[I]}{KI}\right)} \qquad \text{Eq:29}$$

It is known that

$$v0 = \frac{d[P]}{dt} = k3\ [EA] \qquad \text{Eq: 1}$$

Substituting Eq: 29 in Eq: 1 gives

$$v0 = k3\ \frac{[E]t\,[A]}{(KA+[A])\left(1+\frac{[I]}{KI}\right)}$$

$$v0 = \frac{Vmax\,[A]}{(KA+[A])\left(1+\frac{[I]}{KI}\right)} \qquad \text{Eq: 30}$$

The Vmax in non-competitive inhibition reduces by a factor of $\left(1+\frac{[I]}{KI}\right)$. The dissociation constant of the substrate is does not change. Increasing the substrate does not have any effect on inhibition.

3.4.3 Michaelis-Menten approach in gene regulation kinetics

Controlling the timing, location and level of gene expression is essential for the regulation of biological processes. Mathematical modeling of gene regulation kinetics plays a prominent role in probing genetic regulatory networks. Quantitative models can prove or disprove assumptions regarding the process and helps to assess the relationships between behavior of the whole system and that of its component parts. Hill's function is used to model the promoter binding of transcription activators or repressors (Rossi FMV, Mol. Cell. 2000). Michaelis-Menten is an alternative approach which involves solving rate equations representing successive individual steps of the biochemical reaction. An analogy can be drawn between enzymes and transcription factors.

Michaelis-Menten approach is used to model bacteriophage Lambda gene regulation. The bacteriophage lambda has a set of seven promoters that govern the expression of different set of genes during its lifecycle. Two of the promoters are P_R expresses the replication genes as well as the anti-repressor, Cro, the transcriptional activator CII, and the anti-terminator, Q protein and P_{RM} expresses the repressor gene, cI, to maintain lysogeny. In a lysogen, the level of cI increases, P_R is repressed and P_{RM} is occupied by RNA polymerase at a high level, even though the binding affinity of RNA polymerase for P_R is higher than that for P_{RM}. This reflects the competitive nature of cI and RNA polymerase binding (Shea MA and Ackers GK, J. Mol. Biol 1985).

DNA damage response by LexA in E. coli is studied in a synthetic construct with GFP reporter for eight of the SOS operons under negative control by a single repressor LexA. RecA, sensing DNA damage binds to single-stranded DNA gets activated and mediates LexA autocleavage. The drop in LexA levels causes the de-repression of the SOS genes. Once damage has been repaired or bypassed, the level of activated RecA drops, LexA accumulates and represses the SOS operons, and the cells return to their original state. DNA damage response is modeled by using a simple binding of the repressor (LexA) to a regulatory DNA site in each operon, resulting in a Michaelis–Menten form,

$$Xij(t) = \beta i/(1 + \frac{Aj(t)}{ki})$$

where $Xij(t)$ is activity of promoter I in experiment j, $Aj(t)$ is the effective repressor concentration in experiment j, βi is the production rate of the unrepressed promoter, and ki is the effective affinity of the repressor. With this approach the temporal transcriptional program and the concentration profile of the regulatory protein of SOS DNA repair system are determined (Ronen M *et al*., PNAS, 2002).

A rigorous mechanistic representation of *in vitro* RNA synthesis by T7 RNA polymerase is done on the basis of genomic sequence information. A functional dependence of transcript synthesis rate is derived based on concentrations of T7 RNA Polymerase, its promoter, substrate nucleotides, and byproducts that might inhibit the polymerase. The stability behavior of the T7 RNA polymerase is expressed by a time-dependence of *V*max, the maximum rate of transcript formation. The half-life of T7 RNAP activity is calculated to be 50 min *in vitro* conditions (Arnold S, Biotechnol. Bioeng, 2001).

A statistical approach that embeds the deterministic Michaelis–Menten kinetics of gene regulation is developed to study the activity of a transcription factor from gene expression data of the target genes in its regulatory module. The model reconstructs the transcription factor profile using the gene expression profiles of its targets in a Single Input Motif regulatory module. *Streptomyces coelicolor* bacterium, produces many naturally derived antibiotics, antitumor agents and immunosuppressants. The genes coding for antibiotics are clustered in different locations and clustered with calcium-dependent antibiotics regulated by transcriptional activator CdaR. The activity profile of the CdaR regulator is reconstructed studying the profiles of all 17 regulated by CdaR (Khanin R *et al*, Biometrics, 2007).

Though there has been huge progress in dissecting eukaryotic transcriptional mechanisms, fundamental questions remain about the different levels at which transcription is regulated. Eukaryotic transcription is complex and includes multiple mechanisms. To comprehend *in vivo* transcription, its physical context, inside a crowded nucleus in which many genes are being transcribed at once, has to be taken into consideration. The nuclear substructures might help to organize and control gene expression. Models of transcriptional regulations weigh up on transcription factors, chromatin modifications and positional signals. The mathematical modeling of gene regulation kinetics helps characterizing the transcriptional program and helps in understanding embryogenesis, tissue engineering and cellular differentiation.

4. MATERIALS AND METHODS

4.1. Materials

4.1.1 Yeast Transformation

1. Lithium acetate Tris buffer (LiAc-Tris buffer): 100 mM Lithium acetate in 10 mM Tris-HCl pH 7.5.

2. Wash buffer: 10mM Tris-HCl pH 7.5.

3: PEG solution: PEG4000 dissolved in 1:1 (w:v) ratio in LiAc-Tris buffer.

4. Carrier DNA: Salmon sperm DNA (Sigma) is denatured for 10 min at 100°C and immediately put on ice. Frequent denaturation is recommended.

5. YPAD: yeast extract 1%, peptone 2%, Adenine sulphate, dextrose2%.

6. Selection plates to select the transformants: yeast nitrogen base (FORMEDIUM) 0.69%, glucose (2%), agar (2%) and 100ml of 10X amino acid drop-out (FORMEDIUM) solution in 1lt media.

4.1.2 Genomic DNA Extraction

1. 1.2 M SCE buffer: 1.2M Sorbitol, 0.1 M NaCl, 75 mM EDTA, pH 7.0.

2. Lysis buffer (1x): water 7.5 ml, 1 M Tris-HCl (pH 9.7) 1.0 ml, 0.5M EDTA (pH 8.0) 1.0 ml, 10% SDS 0.5 ml). Store at room temperature.

3. Cell wall lysing enzyme: Lyticase 10000 U (Sigma) dissolved in 50 % glycerol, 50% PBS to give a final working stock of 100U/µl . Store at -20° C.

4. Ammonium acetate: prepare 7 M solution in water and adjust pH to 7.0

5. Wash buffer: 10mM Tris pH 7.5

6. RNase A solution: RNase A is added to Tris-HCl 10 mM pH 7.5, NaCl 15 mM to make a working stock of 10U/ µl. Store at -20°C.

7. YPAD: yeast extract 1%, peptone 2%, Adenine sulphate, dextrose2%.

4.1.3 Southern Blotting and detection

1. TBE buffer (5X): 53 g of Tris, 27.5 g of boric acid, 20 ml of 0.5 M EDTA and made upto 1lt with water (pH adjusted to 8.0).

2. Agarose gel: 0.5% agarose is prepared in TBE buffer (1X) and heated until completely melted. On cooling 0.5µg/ml Ethidium Bromide is added before pouring the gel.

3. Nylon hybridization membrane (e.g., Hybond N+, Amersham International, Amersham, UK).

4. Depurination buffer: 500 ml of 0.25 M HCI. Store at room temperature.

5. Denaturation buffer: 1000ml solution comprising of 1.5 M NaCI and 0.5 M NaOH. Store at room temperature.

6. Neutralization solution: 1000ml solution comprising of 1.5 M NaCl and 0.5 M Tris-HCl, pH 7.0. Store at room temperature.

7. Transfer buffer: 1000ml solution comprising of 1.5 M NaCl, 0.25 M NaOH.

8. 20X and 2X standard saline citrate (SSC): (3 M NaCI, 0.3 M trisodium citrate, pH 7.0).

9. UV-transparent plastic wrap

10. Whattman filter paper 3MM

11. 0.2 M EDTA is used to stop labeling reaction.

12. Maleic acid buffer: 1000ml solution comprising of 0.1 M Maleic acid, 0.15 M NaCl, pH 7.5. Store at room temperature.

13. Washing buffer: 0.3% (v/v) Tween 20 added to Maleic acid buffer. Store at room temperature.

14. Detection buffer: 500ml solution comprising of 0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5.

15. Blocking solution: 10X Blocking solution in maleic acid buffer in ratio of 1:10. This solution must be freshly prepared.

16. Antibody solution: Anti-digoxigenin-AP 1:10 000 (75 mU / ml) in blocking solution. This solution must be freshly prepared.

4.1.4 Inducer stocks

1. Estradiol stock: 5M Estradiol is made in 99% ethanol. From this 200 µM stock is made in DMSO. 5 µM stock is always made fresh from the 200 µM DMSO stock. 5M stock in 99% ethanol and 200 µM DMSO stock can be stored at -20° C. Adding 1 µl of this 5 µM estradiol stock in 1ml media makes the final concentration 1nM. A series of concentrations of estradiol is made from 0-200 nM from the same stock.

2. Doxycycline stock: 5M doxycycline stock is made in 50% ethanol. Further dilution stocks 0.05M and 0.005M of doxycyclin are used to make a series of doxycyclin concentration from 0-2 µM. All these stocks can be stored at -20° C. **LIGHT PROTECTION**

4.1.5 Beta Galactosidase CPRG assay

1. Buffer 1: 2.38g HEPES, 0.9g NaCl, 0.065g L-Aspartate, 1g BSA, 50 µl Tween 20 and make up to 100 ml, pH 7.3. Store at 4° C

2. Buffer 2: 27.1 mg of CPRG (chlorophenol red-beta-D-galactopyranoside) in 20ml of Buffer 1. Buffer 2 should be prepared fresh. It can be stored at 4° C up to 2 -3 weeks. Older Buffer 2 turns red.

3. Zinc Chloride solution: 100ml of 3 mM ZnCl2 is prepared in water.

4.2 Methods

4.2.1 Cloning

To study and quantitate the degree of inhibition synthetic construct are designed and cloned into pBluescript-based pRS series of vectors, with

a) Origin of Replication which aids to propagate in E. coli,
b) Selectable bacterial marker (amp, kan),
c) Selectable yeast marker (URA3, LEU2,etc)
d) Multiple cloning site (MCS)

There are two types of pRS available.

i) YIp: Yeast integrative plasmids, which integrate into a particular locus on the yeast chromosome by homologous recombination.

ii) YCp: Yeast centromere (CEN) containing plasmids, which centromeric sequence and yeast Origin of Replication (ARS). They act as chromosomes and segregate during mitosis and meiosis.

Design of Circuits

Components of the CIRCUIT

1. Integrative Sequence:

   The DNA sequence of the yeast chromosome, where the circuit is integrated. This sequence should have a unique restriction site in the plasmid which linearizes DNA for transformation.

2. Promoter Sequence

The DNA sequence that has UAS (Upstream Activating Sequence) and TATA box. This recruits the RNA polymerase II to start Transcription. GAL1UAS is used.

3. Reporter gene sequence

   The ORF (open reading frame, gene) that is to be studied. (e.g.: GFP, lacZ, etc.). It starts with start codon ATG and ends with any of the termination codons TAA, TGA and TAG. This must be followed by a terminator sequence.

4. Inhibitor binding sequence

   This is a DNA sequence to which the inhibitor comes and binds that leads to inhibition of gene expression. Tet operators are used to which chimeric tetR-inhibitor proteins bind.

In the constructs that we used for our research, there is Gal1 promoter with UAS (upstream activating sequence that has four binding sites for Gal4p), no Mig1p binding sites and TATA box. Either upstream of the PGal1nr (Non repressive Gal1 promoter as it lacks Mig1p binding sites) there are tet operators or downstream of the gene and the terminator there are tet operators or both. The activator used is GEV the repressor used is tetR-Ssn6 and the silent information regulator used is tetR-Sir3. GEV is induced by estradiol and binds to Pgal1nr leading to expression of the gene. GEV is integrated into the MRP7 promoter and tetR-Ssn6 and tetR-Sir3 are integrated into the promoter RET2 which are strong and constitutive promoters of the yeast genome.

4.2.2 Bacteria strains and growth

The sequences are obtained by PCR from genomic DNA and cloned into MCS of pRS vectors. Once the cloning of the sequences is done, they are transformed into bacteria, SURE cells (Stratagene; Genotype e14-(McrA-) Δ *(mcrCB-hsdSMR-mrr) 171 endA1 gyrA96 thi-1 supE44 relA1 lac recB recJ sbcC umuC*::Tn*5* (Kanr) *uvrC* [F′ *proAB lacI*qZΔ*M15* Tn*10* (Tetr)]) to amplify the plasmid. Bacteria are grown in LB+Amp (75µg/ml). Plasmid extraction is done using kits from SIGMA. The resultant plasmids are checked by restriction analysis followed by

sequencing. Once the sequence is confirmed then it is integrated into the yeast to perform further studies.

4.2.3 Yeast strains and growth

The S288C cogenic derivatives, BY4741 (MAT A his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; *gal4::kanMX*) and BY4742 (MAT α his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; *gal4::kanMX)* are used in the studies. All reporter constructs are integrated into MAT A cells and the activator and inhibitor constructs are integrated into MAT α strain. Gal4p deleted strains are used to prevent transactivation of the reporter genes.

Cells are grown in minimal medium supplemented with 2% (w/v) glucose for 6 h after induction with estradiol and doxycyclin, starting with 0.05 OD_{600}.

4.2.4 Yeast Transformation

1. The yeast strain to be transformed is inoculated overnight in YPAD.
2. The overnight inoculum is diluted in such a way that the O.D at 600nm reaches 0.6-0.8 after at least three generations (i.e. 4.5 hrs). The volume of the YPAD media should be (n+1)*5.

   n= number of plasmids to be transformed

   1= control

   100mM Lithium acetate is prepared in Tris pH 7.5.

3. PEG4000 is prepared in 100 mM LiAc-Tris buffer in 1:1 = g: ml ratio.
4. In case of plasmid, plasmid is digested with a unique enzyme that cuts only in the integrative sequence of the plasmid (see Note 1). In case of centromeric plasmids and PCR products, restriction digestion is not required.

5. After the culture reaches the required O.D, spin the culture and wash with 10mM Tris pH7.5.
6. After wash resuspend the pellet in (n+1)*5 ml 200mM LiAc-Tris buffer and keep it on a rocker for 40 min at room temperature.
7. After optimal digestion of the plasmid, add 5µl of carrier DNA (Denatured Salmon sperm DNA).
8. After 40 min incubation of cells in LiAc-Tris buffer spin them at 3500 rpm for 10 min and remove the supernatant. Resuspend the cell pellet in (n+1)*200 µl of 200mM Lithium acetate Tris and add to the above plasmid digestion mixture and incubate for 5 min at room temperature.
9. Add 300 µl of LiAc-Tris PEG solution and incubate for 5 min at room temperature.
10. Heat shock is given at 42° C for 15 min.
11. Spin the cells at 10,000 rpm for 1 min and remove the supernatant.
12. To the cell pellet add YPAD and grow them for 45 min at 30° C.
13. Spin the cells for 5 min, remove the supernatant, add 100 µl of 10 mM Tris and plate them on the appropriate selection plates.

4.2.5 Genomic DNA extraction

Method:

1. Prepare the overnight inoculum in YPAD or minimal medium.
2. From this overnight inoculum, inoculate into 5 ml of YPAD or minimal medium grow for 3-5 hrs and the O.D600nm must reach 0.6-0.8.
3. Spin the cells and wash them with 10 mM Tris pH7.5.
4. After wash, resuspend the cells in 150 µl of 1.2 M SCE and add 1 µl of Lyticase 100U/µl. Incubate at 37° C for 60 min (see Note 2).
5. To this add 500 µl of lysis buffer and incubate at room temperature for 5 min.
6. Add 360 µl of 7 M Ammonium acetate pH 7.0 and incubate at room temperature for 10 min.

7. Incubate at 65° C for 10 min and immediately put it on ice for another 10 min.
8. Add 650 µl of chloroform and vortex.
9. Spin the cells and transfer the supernatant carefully into a 2 ml eppendorf.
10. Add 1 ml isopropanol and incubate at room temperature for 15 min.
11. For Southern blotting extracted genomic DNA should be treated with RNAase in TE buffer pH 7.0 and incubate at 37°C for 30 min, to avoid nonspecific signals.
12. To the above mixture add 20 µl 3M Sodium acetate and 450 µl ethanol and incubate at -20°C for 60 min.
13. Spin for 10 min and discard the supernatant. Wash the DNA pellet twice with 70 % ethanol and dry. During washes care should be taken not to lose the pellet.
14. Dissolve the genomic DNA pellet in 50 µl 10 mM Tris pH 7.5 or water (see Note 3).

4.2.6 Southern Blotting and detection

After transformation of the constructs into specific loci on the chromosome, correct integration has to be checked. One way of checking this is by PCR. Primers are designed in such a way that One of the primers is from the chromosome and the other from the construct that is integrated. This could be done both on the 5' end as well as 3' end of the construct. The length of the products obtained would determine the correct integration, but not the copy number of the construct. Southern blotting is a technique that would determine integration and also single or multicopy integration.

This quantitative method helps to find out the copy number of the integrated lacZ sequence, copy number of the activator that drives lacZ gene expression and also copy number of the repressor that drives the repression of lacZ gene.

It involves blotting the membrane with digested genomic DNA, labeling the probe, hybridization of probe to the membrane and detection of target sequence with the labeled probe. In our research DIG High Prime DNA Labeling and Detection Starter Kit II from Roche Applied Science is used.

1. Digest the genomic DNA with appropriate restriction enzyme(s). Load the digested genomic DNA, marker DNA and positive control in an agarose gel. Stain with ethidium bromide.
2. The agarose gel is rinsed in distilled water and then depurinated with 0.25M HCl slowly on a platform shaker for 30 min at room temperature.
3. Discard HCl and rinse the gel with distilled water. Treat the gel with denaturation solution for 20 min at room temperature.
4. Discard denaturation and rinse the gel with distilled water. Treat the gel with neutralization solution for 20 min at room temperature.
5. Place a stack of 8-10 paper towels. Over this place 6-8 dry Whatmann 3MM papers, two Whatmann 3MM papers treated with 2X SSC, Nylon membrane pretreated with 2X SSC, agarose gel (avoid air bubbles while placing), saran wrap with a window slightly smaller than the gel size, two Whatmann 3MM papers treated with 2X SSC and two dry Whatmann 3MM papers in the order mentioned (see Note 4 & 5).
6. With Whatmann 3MM paper form a bridge between the gel and the 20X SSC reservoir, place a glass plate over this to maintain things in place. Leave this for overnight.
7. Carefully disassemble the set up and wrap the membrane in a UV transparent plastic wrap. Irradiate with UV light the membrane on the side with DNA for 1 min, 1.5J/cm^2. Membranes could be used immediately for detection or can be stored at 2-8°C (see Note 6).
8. Denature 1 µg of probe DNA (200-1000bp) that is specific only to the construct integrated into the chromosome, by heating in a boiling water bath for 10 min and quickly chilling on ice. Probe should be highly pure.
9. DIG label the probe with DIG-High prime for one hour or overnight at 37°C. To stop the reaction, add 0.2M EDTA.
10. Pre-hybridize the membrane with hybridization solution for 30 min at 37-42 °C.
11. Add DIG-labeled DNA probe and the hybridization solution to the membrane and incubate overnight.
12. Discard hybridization solution with probe and rinse the membrane with washing buffer.
13. Incubate the membrane in blocking solution for 30 min.
14. Discard blocking solution and incubate the membrane in Antibody solution for 30 min.

15. Pour off the antibody solution and rinse the membrane twice with wash solution. mproper washings would give spotty background.
16. Incubate membrane with detection buffer for 5 min and discard the buffer.
17. Spread the detection reagent over the membrane and leave for 5 min at 20-25°C (see Note 7).
18. Expose the membrane to the suitable imager.

Once the integration of the construct is confirmed, functional analysis of the gene is performed. Preliminary screening is done to check the copy number of the constructs integrated, whether it is the activator, inhibitor or reporter gene. The reporter genes used in our research are GFP (Green Fluorescent Protein), YFP (Yellow Fluorescent Protein), Cerulean (Cyan Fluorescent Protein) and lacZ expressing β-Galactosidase. GFP and YFP are measured by Flow cytometry, YFP and Cerulean by Fluorescence microscopy and β-Galactosidase by CPRG assay.

4.2.7 Flow cytometry

Flow cytometry is a technique used to measure fluorescence intensity of the cells. Cells are carried to the laser intercept in a fluid stream. Here the fluorescent cells scatter laser light and scattered and fluorescent light is collected by lenses, which are steered to the detectors. These detectors produce electronic signals. The advantage of using FACS is evaluation of single cells in large cell samples. Multiple fluorescence parameters are available. Detection threshold being high becomes the limiting factor. In our research, Beckmann Coulter CYTOMICS FC 500 flow cytometry system is used. CXP software is used for generating data.

1. Make overnight culture of the strain that has to be analyzed by flow cytometry. Grow at 30° C. Measure the OD_{600}.
2. Prepare the media 5ml each in different falcon tubes with different concentrations of estradiol and doxycyclin (see Note 8).
3. Grow the cells for 6 hrs, starting with 0.05 OD_{600} (see Note 9).

4. After 6 hrs of growth transfer 1ml of cells into FACS tubes and keep the cells on ice. Samples once kept on ice must be measured within 30-45min.
5. Run the Cleaning protocol with 0.5% bleach followed by water (see NOTE 10).
6. Before sample acquisition cytometer voltages and gains are adjusted in the Cytometer Control panel. The parameters used are :

PARAMETER	VOLTAGE	GAIN
FS (Forward Scatter)	790	5
SS (Side Scatter)	70	1
FL1	490-550	5

Table 3: Parameters of Flow cytometry

Standardize the voltages of FS, SS and FL1.

7. Because of multi carousel loader, 32 tubes can be loaded and each tube is individually and automatically vortexes before sample acquisition. The flow rate must not exceed 3,000 events per sec.
8. Mean fluorescence is obtained from the histogram of SSlin *versus* FSlin with gating. 5–15% of the total cell population is selected.
9. Using CXP Analysis software µsoft Excel sheets can be derived with Mean Fluorescence. GFP fluorescence is directly proportional to the gene expression. Mean Fluorescence of 20,000-30,000 cells is measured.
10. A control where there is no expression of GFP is measured (C). Expression of every sample with varied estradiol (e) and doxycyclin (d), ($F_{e,d}$) is subtracted with the control. Fold inhibition is the ratio of background subtracted expression with and without dox ($F_{e,d}$ –C)/ ($F_{e,0}$ –C). Fold inhibition – 1 reflects the characteristics changes in repression when inhibition is weak (see Notes 11-16 for screening reporter and regulators).

4.2.8 Fluorescence Microscopy

The fluorescence microscope is a technique which works on the principle that certain substances (fluorophores), when irradiated with the light of a specific wavelength, emit energy of higher wavelenght, detectable as visible light. The microscope has a filter that only lets through radiation with the particular wavelength that matches your fluorescing material. When the radiation collides the atoms, electrons are excited to a higher energy level. When the electron falls back to the ground state they emit light of lower energy and has a longer wavelength. The excitation channel is separated from the emission channel with a second filter. GFP, YFP and CFP are the fluorescent reporters that are used in the study. Each of them has different Excitation and Emission wavelengths.

For performing multicolor experiments, fluorescent proteins that are spectrally distant should be chosen. Fluorescence microscopy can be combined with Differential interference contrast (DIC) to enhance contrast.

Zeiss Observer. Z1 inverted microscope is used. Carl Zeiss lens is used. AxioVision 4.6 is the software used to obtain images and mean fluorescence data. This software has Multidimensional Acquisition tool helps to capture the image using more than one channel.

1. Making the overnight, inoculation of overnight culture into media of different concentrations of estradiol and doxycyclin starting with 0.05 OD_{600} and growing the cells at 30° C is similar to that in flow cytometry protocol.
2. After 6 hrs of growth, cells are spun down at 4° C and concentrated to 500 µl (see Note 17).
3. One – two µl of cells are pipetted on the glass slide and covered with cover slip without air bubbles. First take the cells which have maximum expression.
4. Switch on the microscope and the computer connected to it and set the light flow to the camera.
5. The 63X objective could be used to obtain focus. The 100X objective could be used to get a better magnification. With high power objective immersion oil should be used (see Note 18).

6. With AxioVision 4.6 Multidimensional Acquisition tool helps to capture the image using more than one channel. Go to WORK AREA or ACUISITION and click on Multidimensional Acquisition.
7. GFP and DIC channels are chosen. In case of two color experiments YFP, CFP and DIC channels are chosen as YFP and CFP are spectrally distant.
8. Keep in AUTO mode and click on MEASURE. This will give a well exposed image and automatically calculates the exposure time. This step is repeated 3-4 times to get the mean exposure time (see Note 19).
9. Once the exposure time is known, set to FIXED mode, enter value in TIME box and click on MEASURE.
10. Checking the AUTO LIVE box enables continuous exposure time, white balance and color saturation adjustment in the live image.
11. On FIXED mode, place the glass slide with cell sample onto the lens and click on START. This will generate images from all the channels and shows an overlay.
12. Clicking on RUN PROGRAM opens RUN AUTOMATIC MEASUREMENT PROGRAM window. Choose OPEN IMAGES and unclick automatic. Choose the image file that is just created and click on EXECUTE.
13. SEGMENTATION window opens, where TOLERANCE and EDGE SIZE can be adjusted. Adjust Color Saturation clicking on ADVANCED. Once adjusted click on CONTINUE.
14. INTERACTIVE EXECUTION window opens which allows measurement of morphological parameters by drawing contours interactively and marking points relatively to user defined coordinate system. One can discard bad cells or cells that are not in complete frame. Note the measurement of gray background and click CONTINUE.
15. Data files of fluorescence values of individual cells are generated as _Regs.CSV extension file. Save the data files and the image files. Collect data for at least 300 cells from each induction condition.
16. Subtract the fluorescence value of individual cells with gray background measured during interactive execution, this minimizes background effects.

4.2.9 Beta Galactosidase assay with CPRG

The β-Galactosidase enzyme is encoded by the bacterial lacZ gene and converts β-galactosides into monosaccharides. The enzyme is extremely stable, resistant to proteolysis, and easily assayed. CPRG (chlorophenol red-β-D-galactopyranoside) is broken down by β-Galactosidase into galactose and the red colored chlorophenol red, whose absorbance is measured at 595 nm. The detection of lacZ expression by CPRG is 10 times more sensitive than by ONPG (o-nitrophenyl-β-D-galactopyranoside). In our experience, gene expression can be measured with the same sensitivity and dynamical range with the combination of lacZ and CPRG as with quantitative real-time PCR.

1. Inoculate the appropriate minimal medium for overnight growth.
2. Dilute the overnight medium into a fresh medium and grow for 3 to 4 cell division so that the final cell density, as measured by O.D at 600nm, is between 0.4 and 0.8.
3. Pellet 1.5 ml of cells in microcentrifuge tubes at 13,000 rpm for 1 min. Wash the pellet with cold Buffer 1 (4° C) and spin the cells.
4. Resuspend the cells in 0.3 ml of Buffer 1. Now the concentration factor is 5 because the 1.5 ml is concentrated to 0.3 ml.
5. Remove 0.1 ml and dilute into 1 ml of water to measure the OD at 600 nm.
6. Take 0.1 ml of the remaining 0.3 ml in a screw capped tube and freeze-thaw for 3 - 4 times in liquid nitrogen and 37° C water bath, to break open the cells.
7. Add 0.7 ml of cold buffer 2 (4° C) and mix thoroughly by vortexing. Add 1ml of buffer 2 to separately to two tubes which will be the blank during spectrophotometric measurements.
8. Pre heat the water bath to 37° C as the reaction should be carried out at 37° C.
9. Start countdown from the time buffer 2 is added and the tubes are transferred to 37° C and stop when the color of the samples changes from yellow to dark red or brown stopping the reaction with 0.5 ml of 3 M ZnCl2. The blanks should also be treated in the similar manner as the samples (see Note 20 & 21).
10. Spin the cells at 14,000 rpm for 1 min to pellet cell debris.
11. Transfer supernatant to fresh tubes and measure the OD at 595 nm.
12. Calculate β-Galactosidase units with the following formula.

β-Galactosidase units = $1000 * OD_{595} / (t * V * OD_{600})$

t = time of the reaction from adding buffer 2 to adding ZnCl2 to stop the reaction

V = 0.1 * concentration factor (here it is 5, step 4)

4.3. Notes

1. Treatment of the digested plasmid with alkaline phosphates would yield more number of transformants.
2. Incubation with Lyticase for longer time would give better lysis. Lysed cells are more viscous. Yeast cells grown for long time give problems with lysis as they rebuild their cell walls. If more amount of genomic is required, increase volume of the culture but not the cell density.
3. Genomic DNA pellet could be dissolved in higher volume of water and later concentrated. Dissolving the DNA pellet for one hour at 42° C and then at 4°C overnight would result in complete dissolution.
4. Wrap the whole set of Whatmann papers, membrane and gel in saran wrap and make sure that there is no short-circuiting of 20X SSC from the reservoir.
5. Air bubbles between membrane and gel must be avoided, as they can reduce the efficiency of transfer. Gel should be handled with care that it does not break into pieces.
6. Care should be taken to avoid complete drying out of the membrane as it hinders the binding of antibody. Membranes that would not be used immediately should be drained off any liquid, wrapped in a saran wrap and stored at 4°C.
7. Incubation of membrane with detection reagent for longer period gives false positives. The mentioned time of incubation with detection reagent must be followed strictly.
8. When preparing a series of concentrations of the inducers, it is better to use from the same working stock to avoid dilution errors. Pipettes must be well standardized to avoid pipetting errors.

9. The samples must be continuously shaken to avoid settling down, during growth. They are grown for 6 hours in order to reach steady state.
10. Always clean the flow cytometer before and after use. As a control run double distilled water and empty medium to check for contaminants in the flow cytometer.
11. Gating option reduces the background in the samples, focuses on a particular subpopulation and will improve the data.
12. The copy number is based the expression value. Eight different colonies are selected and screened. Single copy integrations are more frequent than high copy. The higher copies are generally whole number multiples of single copy.
13. High copy of activator strain is more reliable as it is less sensitive to fluctuations in estradiol concentrations. At maximum induction it reaches saturation of expression. While screening the activator use different concentrations of the inducer and check the linearity of expression with concentration.
14. High copy of the reporter construct can be used in the cases where there are no long-range interactions.
15. It is observed that the terminator sequence downstream of reporter gene influence gene expression. Three different terminators (Terminators of ACT1, ADH1 and CYC1= T_{ACT1}, T_{ADH1} and T_{CYC1}, respectively) are used in different constructs. Considering GFP expression with T_{CYC1} as x, the expression with T_{ACT1} is 1.3x and with T_{ADH1} is 0.7x.
16. Screening of inhibitor is done with and without repressor inducer, using a series of different concentrations of activator inducers. The ratio of normalized uninhibited expression to inhibited expression will give rise to fold inhibition by the inhibitor.
17. Prior to microscopy measurement, samples could be measured on flow cytometry to check linearity of expression, pipetting errors during preparation of media with inducers. Scattering of cells in flow cytometer also helps to indicate presence of anomalous cell population.
18. While taking measurements care should be taken that cells are fixed and not floating. It is better to have a single layer of cells; avoid cells over cells, as the background may get affected.

19. While measuring to calculate mean exposure time, multiple exposure of the same slide might lead to photo bleaching and give erroneous exposure time. The mean fluorescence should not cross 3000 units.
20. If an intense product color appears quickly, reduce the time of reaction or use a diluted sample of disrupted cells. Do not forget to include this dilution factor in the formula.
21. If there is no color development repeat freeze thaw. Lack of lacZ expression due to mutations in the lacZ open reading frame, misintegration of the construct or low transformation efficiency might also be the reasons for no color development. It is always good to have a positive control to ensure there is no problem with the reagents.

5. RESULTS

5.1 Publication: Synegry of Repression and Silencing Gradients Along the Chromosome

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Synergy of Repression and Silencing Gradients Along the Chromosome

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The expression of a gene is determined by the transcriptional activators and repressors bound to its regulatory regions. It is not clear how these opposing activities are summed to define the degree of silencing of genes within a segment of the eukaryotic chromosome. We show that the general repressor Ssn6 and the silencing protein Sir3 generate inhibitory gradients with similar slopes over a transcribed gene, even though Ssn6 is considered a promoter-specific repressor of single genes, while Sir3 is a regional silencer. When two repression or silencing gradients flank a gene, they have a multiplicative effect on gene expression. A significant amplification of the interacting gradients distinguishes silencing from repression. When a silencing gradient is enhanced, the distance-dependence of the amplification changes and long-range effects are established preferentially. These observations reveal that repression and silencing proteins can attain different tiers in a hierarchy of conserved regulatory modes. The quantitative rules associated with these modes will help to explain the co-expression pattern of adjacent genes in the genome.

Keywords: *Saccharomyces cerevisiae*; gene network; promoter; *cis*-regulatory function; supercompetitive inhibition

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Introduction

Cells differentiate and adapt to environmental changes through the expression of lineage and stimulus-specific genes, and through the repression of inappropriate genes.[1,2] The degree of repression is determined by the interplay between repressors and activators bound to a chromosomal region.[3–5] Insufficient repression of lineage-specific genes is a frequent cause of partial or unsuccessful reprogramming of differentiated cells into stem cells,[6] which underscores the biotechnological relevance of understanding repression in a quantitative way.

Two mechanisms, gene-specific repression and silencing, account for the majority of known instances of transcriptional inhibitory mechanisms in yeast. The co-repressor Ssn6, along with Tup1, the most intensively studied co-repressor in yeast, form a complex and actively repress nearly 3% of the genes in the yeast genome.[7] These general co-repressors are recruited by sequence-specific repressors to promoters of genes regulated by signals triggered by DNA damage, glucose, hypoxia or mating types.[8–10] The binding of activators to DNA is typically not impeded by Ssn6 or Tup1. They inhibit transcription of the target gene through multiple mechanisms, such as interaction with the general transcriptional machinery and through recruitment of cofactors that deacetylate histones and alter chromatin structure.[11–13]

Silencing is defined as a form of repression that acts at distance and involves the formation of specialized chromatin structures. It stands in contrast to gene-specific repressors, which act at or near the site of transcriptional initiation.[14–16] It has been recognized that both inhibitory processes are mediated, in part, by similar molecular mechanisms, which typically involve nucleosome-modifying enzymes. It is not clear how silencing inhibits expression; it affects a step between transcriptional initiation and elongation rather than the recruitment of the transcriptional activator or the polymerase.[17,18]

Silencing has a role in lineage specification of yeast cells. Silencer sequences flank a chromosomal region

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Abbreviation used: GFP, green fluorescent protein.

encompassing a pair of genes that specify the mating type of the cells. Furthermore, silencing affects genes positioned close to the telomere and exogenous genes inserted into ribosomal DNA arrays. Silencing in these regions is less robust than that at the mating-type loci.[15,19,20] The silencers recruit the Sir2, Sir3 and Sir4 silencing proteins. Sir2 deacetylates histones, providing high-affinity docking sites for Sir3. Sir3 and Sir4 bind to each other *in vitro* with a high degree of cooperativity.[21] The subsequent binding of the Sir2-Sir3-Sir4 protein complex is thought to enable them to spread along chromatin by "sequential deacetylation".[16,22–24] Other mechanisms, such as looping of DNA, have been invoked to explain the action of Sir proteins at a distance.[25]

While quantitative aspects of prokaryotic repression have been studied extensively,[26–31] the principles and quantitative rules of eukaryotic transcriptional repression mechanisms are not clear.

First, we developed a simple model that explains the functional form of inhibition by both Sir3 and Ssn6 when they bind to sites close to the promoter. Next, we explored how inhibition declined as the binding sites were moved away from the promoter. In contrast to our expectations, we found that Ssn6 and Sir3 have similar distance-dependent behaviour. However, silencing was distinguishable from repression when the binding sites flanked a gene. While Ssn6-bound operators had a multiplicative effect on gene expression, the effect of silencing could be explained by a strong synergy due to the amplification of interacting upstream and downstream repression gradients. The amplified gradients have a multiplicative effect on gene expression. These results show how the position and distribution of Ssn6 and Sir3 binding sites correlate with inhibition, which provides a new point of view of how to distinguish promoter-specific repression from silencing.

Results

Repression by Ssn6 when it is recruited upstream or downstream of a gene

We explored the steady-state behavior of Ssn6-mediated repression in the yeast *Saccharomyces cerevisiae* with dual-control gene constructs, where the position of the repressor-binding sites relative to the activator-binding sites was varied (Fig. 1). The

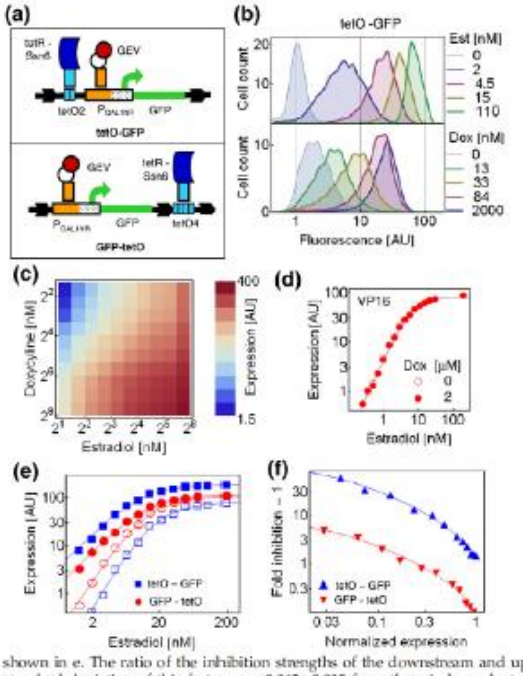

Fig. 1. Effect of upstream and downstream repression sites on gene expression. (a) Upstream (tetO-GFP) and downstream (GFP-tetO) repression constructs. Transcriptional terminators (black arrowheads) serve to prevent transcription from interfering with binding of TetR domain to the *tet* operators. (b) Distribution of total cell fluorescence of PRY438 (tetO-GFP). The estradiol concentration was varied in the presence of 2 μM doxycycline (non-repressive condition; upper panel); and doxycycline was varied in the presence of 6.7 nM estradiol (lower panel). (c) Density plot of expression of the Ssn6 controlled tetO2-GFP construct (PRY432). The expression level, and the doxycycline and estradiol concentrations follow a logarithmic scale, except for the transition to the last column, where 32 nM is followed by 256 nM estradiol. (d) tetR-VP16 has no effect on transcription from a downstream site. Open and filled symbols denote expression at 0 and 2 μM doxycycline (PRY368). Expression induced by rtTA at a tetO7-GFP construct is shown in Supplementary Data Fig. S1 as a positive control. (e) Open and empty symbols denote expression in non-repressive (2 μM doxycycline) and repressive (no doxycycline) conditions (PRY430, 432). (f) Fold inhibition was calculated from the data shown in e. The ratio of the inhibition strengths of the downstream and upstream constructs was 0.078. The mean and standard deviation of this factor was 0.062±0.013 from three independent experiments.

activator GEV binds to a modified *GAL1* promoter, P_{GAL1NR}, which lacks the Mig1p recognition sites (Fig. 1a).[32] GEV is composed of a Gal4p DNA-binding domain, an estradiol receptor domain, and the transcriptional activation domain VP16.[33] Incremental induction of gene expression by estradiol generated a graded response, as measured by the fluorescence of green fluorescent protein (GFP) in a cell population (Fig. 1b, upper panel). Two tetR-binding sites (*tet* operators) were placed upstream of P_{GAL1NR} to serve as docking sites for the tetR–Ssn6 fusion protein. Doxycycline induces the dissociation of tetR from the DNA. In this way, the intensity of transcriptional activation and repression can be adjusted independently. Increasing the concentration of doxycycline results in a graded derepression of gene expression in a cell population (Fig. 1b, lower panel).

Expression was examined over a broad range of estradiol and doxycycline concentrations (Fig. 1c), which enables the detailed mapping of the *cis*-regulatory function of the promoter.[34] TetR-Ssn6 reduced the expression only two- to threefold at maximal gene expression but up to 50-fold at a lower level of transcription activation (Fig. 1e). Repression was slightly stronger when seven *tet* operators were placed upstream of the promoter (Supplementary Data Fig. S1c).

In contrast to higher eukaryotes, yeast transcriptional activators and repressors exert their influence within the promoter and over short distances, typically less than 0.5 – 1 kb.[35,36] We explored the effect of repressor-binding to sites considered to be outside the regulatory region of the gene. For this purpose, four *tet* operators were inserted downstream of the reporter gene GFP, at a distance of 1 kb from the promoter (Fig. 1a). The reporter construct and the *tet* operators were flanked by well-defined transcriptional terminators.[37] No inhibition was observed at maximal gene expression. Surprisingly, inhibition increased up to fivefold at a low intensity of gene activation (Fig. 1e). Binding of the $tetR^r$-VP16 activator to these sites did not repress or activate transcription (Fig. 1d).

When fold inhibition − 1 was plotted against normalized (unrepressed) gene expression (see Materials and Methods), measured in non-repressive conditions, similar profiles were seen for both the

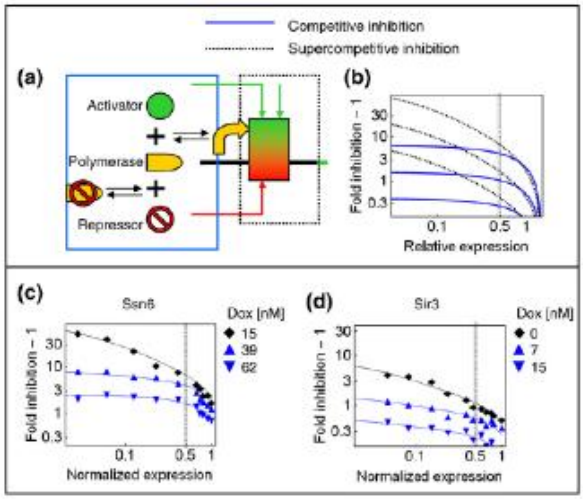

Fig. 2. Competitive and supercompetitive forms of repression. (a) Processes on the promoter result in competitive (enclosed by a blue frame) and supercompetitive inhibition (enclosed by a black dotted frame) of transcription. The amber block arrow denotes the transcriptional initiation complex, and the rectangle filled with a green–red gradient represents the permissiveness of the DNA segment with respect to transcription. (b) Blue lines represent inhibition functions by solving Eq. (5) for three different intensities of competitive inhibition $f_1(R)=0.4$, 1.6 and 6.4 in the absence of supercompetitive inhibition ($f_2(R)=0$). In contrast, $f_1(R)=0$ for the broken black lines, while the value of $f_2(R)$ was set to 0.1, 0.4 and 1.6. The gray line denotes the half-maximal expression. (c) Repression at the tetO2-GFP construct (PRY432). Contributions of competitive, $f_1(R)$, and supercompetitive, $f_2(R)$, forms of repression to the inhibition functions were obtained from fitting Eq. (5) to experimental data. $f_1(R)=3.98$, 5.76 and 5.76 fixed; $f_2(R)=0$, 0.005 and 0.14, when doxycycline concentration was adjusted to 62 nM, 39 nM and 15 nM, respectively. (d) Silencing at the tetO2-GFP construct (PRY351). $f_1(R)=0.33$, 0.81 and 1.3; $f_2(R)=0.002$, 0.006, and 0.037, when doxycycline concentration was adjusted to 15 nM, 7 nM and 0 nM, respectively.

upstream (proximal) and downstream (distant) repression constructs (Fig. 1f). This similarity was confirmed by fitting an inhibition function to the experimental data. The inhibition function incorporates two forms of repression mechanisms (see below). When the inhibition function, fit to the upstream construct, was shifted downwards, it matched the data points obtained for the downstream construct (Fig. 1f). Thus, repression by Ssn6 from the upstream and downstream sites is mediated by kinetically similar mechanisms, and only the respective repression strengths differ (see Theory for the calculation of repression/inhibition strength).

Competitive and supercompetitive forms of repression

The increase in fold inhibition − 1 with decreasing gene activation suggests some form of competition. Competition can occur between the effectors of activators and repressors during the recruitment of the transcriptional machinery (Fig. 2a and b; Theory). When the occupancy of Ssn6 binding sites was reduced by doxycycline, the repression profiles were transformed nonlinearly; the inhibition curves flattened out (Fig. 2c). Fold-inhibition − 1 doubled, at most, when gene activation was reduced from half-maximal to an arbitrarily low detectable value. This behavior is typical of competitive inhibition (Fig. 2b, blue lines).[38]

On the other hand, the value of the inhibition function increased more than twofold in the range below the half-maximal expression, when the occupancy of repressor-binding sites was high (doxycycline = 15 nM, Fig. 2c). We termed this behavior supercompetitive inhibition (Fig. 2b, black dotted lines). This behavior is compatible with models that include a controlled transition between two states of the promoter region: one is permissive, while the other is non-permissive for an initiated transcription (Fig. 2a). The transition between these two states is catalyzed by the mediators of the activator and repressor.

Each inhibition function was fit to experimental data by adjusting the contributions of competitive and supercompetitive repression (see Materials and Methods, and Theory). The contribution of super-

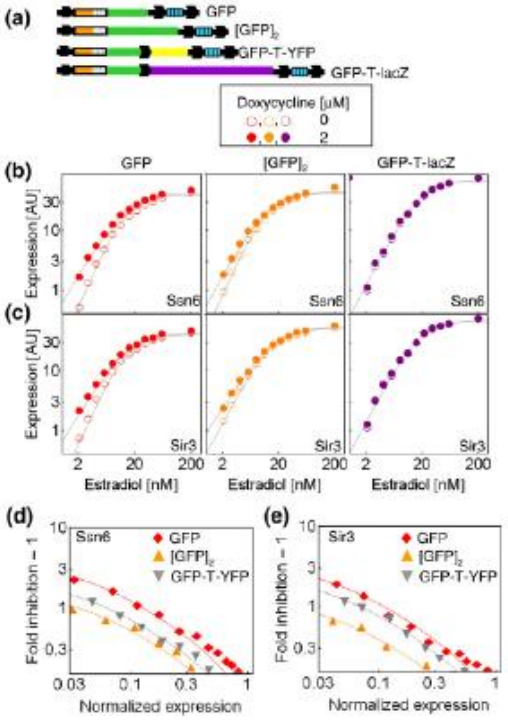

Fig. 3. Distance-dependence of repression and silencing from downstream sites. (a) Reporter constructs with increasing length are regulated by tetR-Ssn6 (PRY418,-419,-420,-421) or with tetR-Sir3 (PRY364,-365,-366,-367). β-Galactosidase activity was not detected in the strains carrying the GFP-T-lacZ construct (Supplementary Data Table S1). (b) and (c) Expression under non-repressive (filled circles) and repressive conditions (open circles). (d) The repression strength relative to GFP is 0.43 (0.33±0.13) and 0.59 for the $[GFP]_2$ and the GFP-T-YFP constructs, respectively. (e) The silencing strength relative to GFP is 0.36 (0.36±0.05) and 0.71 for the $[GFP]_2$ and GFP-T-YFP constructs, respectively.

competitive inhibition is more pronounced at high occupancy of repressor-binding sites (Fig. 2c).

It is interesting that the contribution of an inhibitory mode is not constant as the binding of the repressor is varied. This may reflect the fact that the proportion of mediators recruited by Ssn6 varies as a function of Ssn6 binding site occupancy. Such a relation has been described for transcriptional activators.[39]

When the Sir3 silencing protein was recruited to the operators, the overall inhibition strength was lower than for Ssn6. Although in a less pronounced way, Sir3 followed a similar trend: the contribution of supercompetitive inhibition increased with stronger Sir3 binding (Fig. 2d).

Inhibitory gradients of similar slopes are generated by Ssn6 and Sir3

Constructs were designed to increase the distance between the promoter and *tet* operators by lengthening the reporter gene (Fig. 3a). Duplicating the GFP sequence in a tandem array reduced the strength of inhibition more than twofold (Fig. 3b and c). When the reporter was lengthened to 4.2 kb by inserting a lacZ sequence, repression decreased to below the detection limit.

The unexpected effect of Ssn6 at distance prompted us to compare it with Sir3, which is known to spread along the chromosome.[40,41] When the above gene expression systems were regulated by the tetR–Sir3 fusion protein, the distance-dependent decrease of silencing strength was similar to that of repression (Fig. 3b and c). This indicates that both repression and silencing generate comparable single inhibitory gradients.

Long-range interactions can arise through looping of DNA, as well. Looping was reported to occur between promoters and terminators during transcription.[42] To test whether the terminator-mediated looping accounts for the inhibitory effects of Ssn6 from the downstream sites, a terminator was inserted between the GFP and the YFP sequence (Fig. 3a). The terminators flanking the *tet* operators were retained. In the resulting GFP-T-YFP construct, the *tet* operators were placed at a distance of 1 kb from the transcriptional termination site of the GFP reporter gene (Fig. 3a). This separation is expected to reduce the efficiency of looping-mediated repression. We did not observe a reduction of inhibition strength at the GFP-T-YFP construct in comparison to $[GFP]_2$ (Fig. 3d and e). Rather, the inhibition was stronger, especially with Sir3 (Fig. 3e). The YFP in the GFP-T-YFP construct is a non-transcribed gene. Thus, it is possible that the Sir proteins spread more efficiently along a non-transcribed gene than along a transcribed gene, which would lead to a more efficient silencing of GFP-T-YFP in comparison to the fully transcribed $[GFP]_2$.

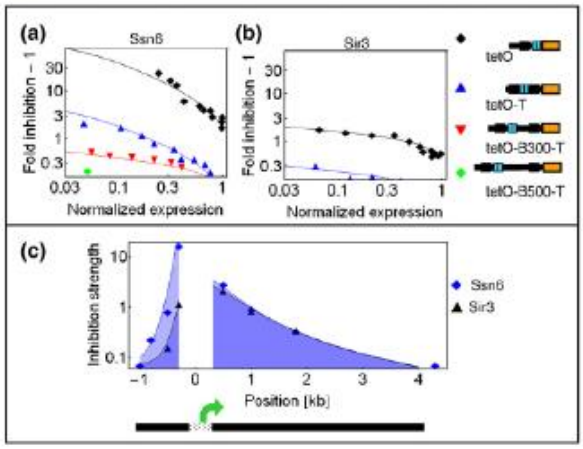

Fig. 4. Reconstruction of the upstream and downstream gradients. (a) and (b) The tetO2 operators were adjacent to P_{GAL1NR} or separated from it by T_{GAL7}, spacer B-300—T_{GAL7}, spacer B-500—T_{GAL7} sequences. The spacer B sequence encompasses part of the open reading frame and the transcriptional terminator region of the *RPN12* gene. Constructs were regulated by: (a) tetR-Ssn6 (PRY438,-475.4,-483.1,-450; and by (b) tetR-Sir3 (PRY351,-474.4). (c) The mean inhibition strengths (see Theory) of the constructs shown in Figs. 3b and c, 4a and b, and Supplementary Data Fig.S2. The ratios of inhibition strengths at the lacZ(1–150) and lacZ(1–450) constructs were used to define the strength relative to GFP. A mean inhibition strength of 0.075 corresponds to the detection limit. The position denotes the distance between the TATA box and the *tet* operators. The decline of the inhibition strength was similar to an exponential function close to the promoter but the decline slowed at larger distances.

To assess the features of the inhibitory gradients in more detail, constructs were designed, in which either the reporter gene was shortened or the sequence separating the upstream *tet* operators and the promoter was prolonged (Fig. 4). Silencing nucleated upstream of the promoter was weak. It displayed an inhibition function comparable to that produced by repression sites having low occupancy (Fig. 4a and b). Inhibition by Ssn6 and Sir3 declined more precipitously upstream than downstream of the transcriptional initiation site (Fig. 4c).

The slope of a gradient is a useful measure of action at a distance because it is independent of the absolute intensity of the effect. While the action at a distance has been considered to be a distinguishing feature of silencing, the slopes of single inhibitory gradients generated by Ssn6 and Sir3 are remarkably similar both upstream and downstream of the transcription initiation site (Fig. 4c).

Silencing nucleated by Sir3 recapitulates the major quantitative aspects of endogenous silencing

Although the spreading of the Sir2-Sir3-Sir4 protein complex is a phenomenon common to silencing at the telomere and the mating-type loci, the molecular mechanisms of the recruitment/nucleation of silencing varies at these loci.[15] We explored if the Sir3-nucleated silencing reproduces characteristic features of the endogenous silencing: the distance-dependence, cooperation of silencers and variegated expression.

Inhibition of gene expression by telomeric silencing drops around 10 times over a distance of 1 kb,[43] which is comparable to the slope of the gradients shown in Fig. 4c.

The silencers flanking the HML mating type locus cooperate. A gene flanked by the HML E and I silencers is repressed up to 50-fold, even though none of them is capable of repressing the gene individually.[44] We compared silencing nucleated by Sir3 to silencing nucleated by the HML E-element (Fig. 5a). The E element alone did not inhibit expression (Fig. 5b). Binding of tetR-Sir3 to the combined tetO-GFP-E silencer construct led to 15-fold inhibition at low concentrations of estradiol. Similar inhibition was observed when both the upstream and downstream silencing was nucleated by Sir3 (Fig. 5a). These findings indicate that silencing nucleated by the E-element and Sir3 behaves similarly with respect to synergy.

Variegated expression has been observed for genes flanked by the mating-type loci silencers, in *sir1* cells.[45,46] The dual silencing construct also displayed a broad bimodal (variegated) expression in a cell population, at intermediate estradiol concentrations (Fig. 5c). The above results show that silencing at the native genomic loci and silencing nucleated by Sir3 have similar distance dependence, cooperative action and variegation of expression.

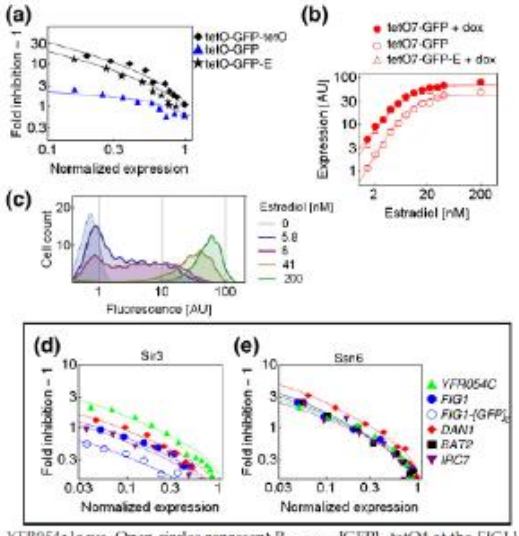

Fig. 5. Characteristic features of silencing nucleated by Sir3. (a) Silencing strength at the tetO7-GFP-tetO4 (PRY372) and tetO7-GFP-E-element (PRY533.10) constructs is 4.6 and 5.2 times higher than the product of inhibition functions of the parent constructs. The upstream parent for both constructs is tetO7-GFP (PRY370). (b) When tetR-Sir3 does not bind to the *tet* operators (in the presence of 2 μM doxycycline), the expression of tetO7-GFP (PRY370) is nearly identical with that of the tetO7-GFP-E-element (PRY533.10). Thus, the degree of inhibition by the E-element alone is very low (<1.5%, compare filled circles and open triangles). (c) Single-cell distribution of gene expression in the tetO2-GFP-tetO4 construct (PRY355) as the estradiol concentration was varied in repressive condition. (d) P_{GAL1NR}-GFP-tetO4 constructs were inserted into the indicated chromosomal loci, in strains with tetR-Sir3 (PRY342,-364,-371,-497.7). The *FIG1* locus displays silencing by a factor of 0.40±0.09 less than the *YFR054c* locus. Open circles represent P_{GAL1NR}-[GFP]$_2$-tetO4 at the *FIG1* locus (PRY453). (e) Strains constructed as in d but with tetR-Ssn6 (PRY379A,-386,-387,-457,-496.7). The *FIG1* locus displays repression by a factor of 0.62±0.04 less than the *DAN1* locus.

Chromosomal position effect is also typical of silencing. Among the loci examined, silencing was strongest at the *YFR054c* locus, positioned 11 kb from the telomere, two- to threefold higher than most of the other loci examined (Fig. 5d). Loci closer to the telomere typically display an even higher degree of silencing.[43] Nevertheless, silencing at the *IRC7* locus, which is 6 kb more telomere-proximal than the *YFR054c* locus, was comparable to that at the *FIG1* locus, located 400 kb away from the telomere (Fig. 5d). This may reflect the discontinuous nature of silencing, which is frequently caused by insulators encountered at telomeres.[47,48]

A genome-wide analysis revealed that hypoacetylated subtelomeric regions are enriched in Ssn6-Tup1 regulated genes.[49] The *BAT2* and *DAN1* loci are found in the subtelomeric domain of chromosome X. A comparison of downstream repression constructs revealed that the *DAN1* locus imparts around 1.6-fold stronger repression relative to the other loci (Fig. 5e). The *DAN1* intergenic locus displays the highest degree of Hda1-dependent deacetylation in the subtelomeric domain of chromosome X,[49] which may explain the pronounced repression at the *DAN1* locus. Furthermore, the *DAN1* but not the *BAT2* gene is strongly derepressed in cells in which Tup1, the interaction partner of Ssn6, is deleted.[50] In summary, silencing can be enhanced at telomeric loci, whereas repression can be enhanced at hypoacetylated subtelomeric loci.

Interaction of repression gradients

Since two silencers show a strong cooperative interaction, we tested if two repression gradients cooperate by combining the upstream and downstream repression sites into dual repression constructs (Fig. 6a). When upstream and downstream constructs with nearly equal inhibition were combined, the resulting dual construct displayed 3.5-fold higher inhibition strength in comparison to that of the parent constructs (Fig. 6b).

When two gradients affect the same component or parallel pathways in the transcriptional repression process, they have an additive effect. Alternatively, when the two gradients affect different components in subsequent stages of a process, they have a multiplicative effect on gene expression. When upstream and downstream constructs with equal inhibition strengths are combined, the additive and the multiplicative mechanisms predict similar inhibition function for the dual repression construct (dual inhibition function) (Fig. 6c). When constructs with dissimilar (weak and strong) inhibition are combined, the two mechanisms become distinguishable. The additive mechanism generates nearly identical inhibition functions for the single strong and the dual repression constructs (Fig. 6c, continuous blue and upper broken gray lines). However, these two functions diverge as the activation of gene expression is reduced, assuming a multiplicative mechanism (Fig. 6c, upper dotted lines).

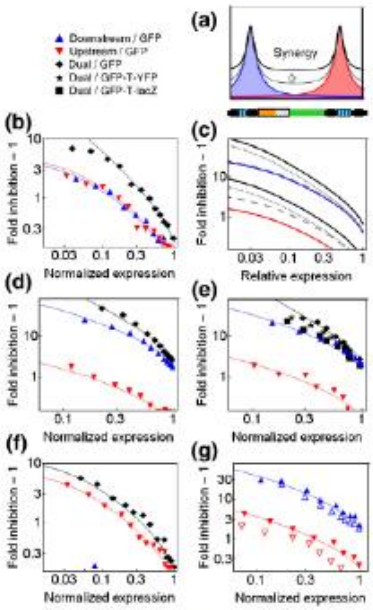

Fig. 6. Combined effect of upstream and downstream repression sites on gene expression. (a) and (b) The inhibition strength at the tetO2-T_{GAL7}-GFP-tetO4 construct (PRY478.1) was $I = 2.3$, while for the upstream and downstream constructs (PRY475.4,-418) $I = 0.57$ and 0.65, respectively. The black line is the constant multiple ($a = 1.3$) of the product of the upstream (blue) and downstream (red) inhibition functions. (c) The broken and dotted gray lines denote the sum and product of two inhibition functions, respectively. Black lines are constant multiples of the gray lines. Interactions were calculated between two red (identical inhibition) curves and between the red and blue (dissimilar inhibition) curves. (d) and (e) tetO2-GFP-tetO4 and its parent constructs were inserted at the *YFR054c* locus ((d) PRY418,-438,-355.1; $a = 1.4$) and the *FIG1* locus ((e) PRY389, 390, 391; $a = 1.48$). The repression strengths of the tetO2-GFP-T-YFP-tetO4 and the tetO2-GFP-T-lacZ-tetO4 constructs (PRY536,-537) fall in between that of the tetO2-GFP and tetO2-GFP-tetO4. (f) The amplification factor for the dual inhibition construct containing tetO2-spacer B-500-T_{GAL7} is $a = 1.59$ (PRY418, -451B,-450B). (g) Open symbols represent the srb10 strains containing the tetO2-GFP (PRY393,-441) and GFP-tetO4 constructs (PRY418,-445). The mean relative differences of repression strength between the srb10 and WT cells are −0.12±0.15 and 0.53±0.14 for the respective constructs.

The multiplicative mechanism fit the experimental data more faithfully, when constructs with dissimilar upstream and downstream inhibition strengths were combined (Fig. 6d).

If one of the sites does not contribute to repression, the dual inhibition function is equal to the inhibition function of the contributing repression site, assuming a multiplicative effect. However, a small amplification was clearly seen when a longer spacer sequence separated the *tet* operators and the P_{GAL1NR} (Fig. 6f). When this upstream construct was combined into a dual repression construct, a 1.6-fold stronger inhibition was observed in comparison to the downstream repression construct, even though the upstream site alone did not repress expression (Fig. 6f). Therefore, the dual inhibition function was approximated by the product of the upstream and downstream inhibition functions multiplied by a constant to account for a small degree of amplification (see Theory; Fig. 6, black lines).

The multiplicative effect was not affected by changing the chromosomal position of the construct (Fig. 6d and e). As the reporter gene was lengthened in the dual repression constructs, the repression strength reduced and converged to that of the single upstream repression construct (Fig. 6e).

The multiplicative effect implies that mutations in some of the mediators of Ssn6 would affect the upstream and downstream repression constructs asymmetrically. When effectors of Ssn6, such as the Hda1 histone deacetylase or the component of the mediator, Srb10, are mutated individually, a variable, usually small, degree of derepression ensues in a subset of target genes.[7,51–55] *HDA1* disruption caused a nearly twofold increase in the absolute expression level of the reporter gene but the relative inhibition strength was unaffected (Supplementary Data Fig. S3). *SRB10* disruption led to a twofold reduction in the inhibition strength in the downstream repression construct (Fig. 6g) while only a 1.2-fold reduction was observed for the upstream repression construct. This asymmetric effect of *SRB10* disruption underscores the multiplicative mechanism at the molecular level.

We also examined if repression gradients could affect expression of endogenous genes. Expression of *FIG1* was reduced twofold when tetR-Ssn6 bound to sites downstream of the 1 kb long *FIG1* gene (Supplementary Data Table S2).

Amplification of the silencing gradients

The synergy of Sir3 nucleated silencing was explored by a construct in which the *tet* operators flanked the expression unit (Fig 7a). The fold inhibition − 1 of the dual silencing construct reached a value of around 10 and up to 30 in some constructs, when the upstream and downstream sites individually reached a value of around 1 (Fig. 7).

The inhibition strength of the dual silencing construct at the *FIG1* locus was 6.6-fold higher than expected from a simple multiplicative effect (Fig. 7b). A strong, approximately fivefold, amplification was observed also in the dual silencing construct where the upstream sites alone did not inhibit expression (Fig. 7c). While the amplification of interacting repression gradients varied between

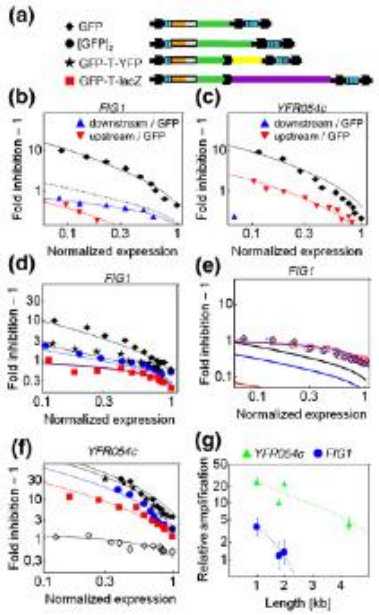

Fig. 7. Amplification of silencing gradients. (a) Dual inhibition constructs with reporter genes of varying lengths. (b) I (mean silencing strength) = 4.3 for tetO2-GFP-tetO4 at the *FIG1* locus (PRY379). I = 0.65 for the product of the inhibition functions (dotted gray lines) obtained for the parent constructs (PRY371,-378). (c) Silencing at the *YFR054c* locus, when the upstream operators are separated by the spacer B-500 from P_{GAL1NR} (PRY364,-423B,-422B). I = 4.15 and 0.8, for the dual construct and the product function, respectively. (d and e) Dual silencing constructs at the *FIG1* locus (PRY379, -434,-435,-436) (d). The upstream silencing constructs with the respective reporter genes (tetO2-GFP, tetO2-$[GFP]_2$, tetO2-GFP-T-lacZ; in the strains PRY378,-461,-462) are denoted by the corresponding open symbols in (e). They behave similarly (scattered around the purple line). Functions for the downstream constructs (black, blue and red lines) are shown in (e). (f) The dual silencing constructs (PRY355,-501.5,-499; YSSD225) and the tetO2-GFP upstream silencing construct were integrated into the *YFR054c* locus (PRY351). (g) Amplification factors (for the strains shown in (d) and (f) were calculated relative to the GFP-tetO4 at the *FIG1* or *YFR054c* locus. The mean and standard deviation of the relative amplification factors were calculated from three independent experiments for GFP, $[GFP]_2$ and GFP-T-YFP and GFP-T-lacZ.

1.3 and 1.6 (Fig. 6), the above values for silencing were considerably higher.

Amplification can arise when an inhibitory gradient enhances the nucleation and/or the spreading

of a neighboring gradient. If one gradient is strengthened, the neighboring gradient spreads more efficiently, provided the effects of Sir3 on transcription and spreading are correlated. This scenario can be tested by plotting the degree of amplification as a function of distance between the *tet* operators that flank the gene expression unit. A more efficient spreading would entail a shallower decline in the amplification as the distance between the flanking operators is increased (Supplementary Data Fig. S4).

The distance dependence of the amplification was tested using the dual inhibition constructs with the GFP, tandem GFP, GFP-T-YFP and GFP-T-lacZ reporter genes (Fig. 7a), at the *FIG1* locus. The silencing strength was reduced more than twofold, when the GFP sequence was duplicated (Fig. 7d). Silencing at the 4 kb long GFP-T-lacZ reporter was not stronger than silencing at the corresponding upstream constructs (Fig. 7d and e).

Next, we examined the synergy of gradients at the *YFR054c* locus (Fig. 7f). The dual silencing construct containing the GFP-T-lacZ reporter showed a considerable silencing at the *YFR054c* locus, at least fivefold stronger than at the *FIG1* locus (Fig. 7d). This indicates that gradients interacting over long distances are amplified preferentially.

When amplification was plotted as a function of distance, it became evident that the decline of amplification was shallower at the telomeric *YFR054c* locus, in comparison to the non-telomeric *FIG1* locus (Fig. 7g).

A more efficient spreading of the Sir proteins between two nucleation sites can be achieved by a stronger deacetylation through Sir2. Typically, Sir2-dependent deacetylation becomes stronger toward the telomere.[54] Furthermore, increasing Sir3 concentrations induce an increasing compaction of the chromatin *in vitro*.[55] Compaction can create contacts between non-neighboring nucleosomes, which shortens the distance between the two gradients. Consequently, the spreading of the silencing factors is facilitated.

Discussion

We employed dual control gene expression systems to explore the quantitative rules of inhibition of gene expression by the Ssn6 repressor and Sir3 silencing proteins. It was surprising to observe repression by Ssn6 from downstream of a coding region, since co-repressors have been thought to have a local effect close to the transcriptional initiation site. Multiple mechanisms could account for such a long-range effect. For example, Ssn6 can affect chromatin organization over a distance of 5 kb.[12] Chromatin organization is influenced by histone acetylation, although in our system Hda1 did not seem to mediate the inhibitory effect of Ssn6. On the other hand, mutation of Srb10 (Ssn3, Cdk8), a component of the mediator complex, led to a reduction of repression from a downstream site (Fig. 6g). It is not clear how a downstream repressor would affect transcription through the mediator, which is thought to recruit the polymerase to the promoter. However, recent studies employing chromatin immunoprecipitation have revealed that Srb10 and other components of the mediator complex can be found in promoter regions and in gene-coding regions[56,57]. It is plausible that the Srb10 bound to the gene-coding region may directly contact Ssn6 downstream of the coding region, which could mediate repression.

Genome-wide studies suggest that chromatin modifications, a hallmark of repression and silencing, have a major impact on the correlation between the expression of adjacent genes.[58] Given the widespread role of the Ssn6–Tup1 complex in repression and its evolutionary conservation, it is plausible that repression gradients contribute to the determination of co-expression patterns of adjacent genes.

Our major goal was to describe repression and silencing in quantitative terms. A model should include the key processes and parameters to reproduce the features commonly observed across the different constructs. With the help of the dual control gene circuit we were able to identify an inhibition function that successfully explains the action of both Ssn6 and Sir3. The inhibition function, which includes competitive and supercompetitive forms of repression, was then used to characterize the distance-dependent action and the synergy. It was surprising that transcriptional inhibition by Ssn6 and by Sir3 can be described by gradients with nearly identical slopes, because silencing was distinguished by its ability to inhibit transcription at distance; and Sir proteins were shown to spread along the chromosome. However, when two gradients interacted, Ssn6 and Sir3 behaved differently.

The inhibition of gene expression by Ssn6 is described adequately by the product of the upstream and downstream repression functions. The multiplicative interaction arises because the components mediating the repression are affected asymmetrically by the upstream and downstream gradients (Fig. 6g). The repression gradients undergo a small, less than twofold, amplification during the interaction (Fig. 6).

The term silencer was coined for a sequence at the mating-type locus that represses gene expression at a distance.[14] Our results refine the definition of silencing regarding its effect on transcription. While both repressor and silencing proteins can generate transcriptional inhibitory gradients of equal slopes, only silencing gradients that flank a gene can undergo significant amplification during their interaction (Fig. 8a). The range of amplification of interacting gradient lengthened, when the gene construct was transferred from a non-telomeric locus to a telomeric locus (Figs. 7g and 8b).

Amplification assumes dispersed nucleation sites that flank a gene. The advantage of dispersed over clustered nucleation sites is also evidenced by comparing constructs where the total number of opera-

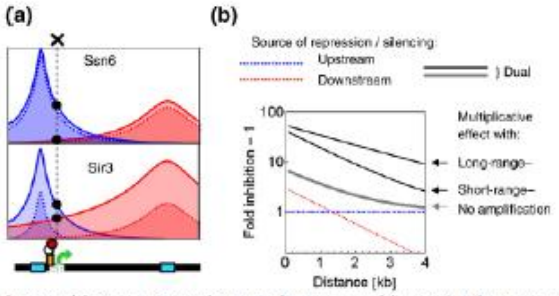

Fig. 8. Hierarchy of regulatory modes in transcriptional repression and silencing. (a) Single upstream and downstream gradients (broken blue and red lines) undergo amplification during interaction (continuous lines). (b) Fold inhibition is shown for a given intensity of gene activation when the position of the upstream repressor-binding site is fixed (dotted blue line) and the distance of the downstream site is varied (dotted red line). When the two sites are combined, they have a multiplicative effect on gene expression. Three scenarios for the amplification of the interacting gradients are shown: no amplification (similar to repression by Ssn6), short-range amplification (comparable to silencing by Sir3 at a nontelomeric locus, *FIG1*, Fig. 7g) and long-range amplification (comparable to silencing by Sir3 at a telomeric locus, *YFR054c*, Fig. 7g).

tors is nearly equal. For example, Sir3 binding to two upstream and four downstream operators produced nearly twofold stronger silencing than binding to seven upstream operators (Fig. 7b; Supplementary Data Fig. S1). The understanding of the formation and interaction of gradients is important to explain the correlations between the expression of adjacent genes, in terms of the steady-state level and fluctuations.[59,60]

Repression mechanisms can attain different tiers in different dimensions of repression modes. These modes comprise the kinetic form of inhibition (competitive and supercompetitive), the slopes of their inhibitory gradients, the additive or multiplicative nature of interaction, and the magnitude and range of amplification during the interaction of the gradients. Ssn6 and Sir3 can be conceived as two points in this continuum of repression modes. Even though amplification is typical of silencing, residual amplification is observed also for Ssn6. The continuous nature of repression modes is supported by observations that a single mutation can convert a repressor into a protein whose chromatin-modifying properties are reminiscent of silencing proteins.[61] Using the approach outlined above, different repressor/silencing proteins can be classified functionally, according to the tiers they reach in the different repression modes.

Such a characterization is essential to the design of biological systems for biotechnological purposes and tissue engineering, such as enhancement of cellular differentiation and reprogramming of differentiated cells into stem cells through regulation of gene expression.[6,62] When the number and the type of activator and repressor-binding sites around a gene of interest are identified, it can be determined which level each repressor will attain in the set of repression modes. This in turn enables, for example, the selection of a particular repressor whose activity when induced, leads to the most efficient repression. Optimal repression of inappropriate lineage-specific genes will then improve cellular differentiation.

Theory

Model of transcriptional repression

The models of prokaryotic gene regulation are typically based on statistical weights of promoter configurations.[27,28] Eukaryotic repression is indirect and includes multiple mechanisms. The model incorporates two forms of antagonism between the activator and the repressor.

Competitive inhibition

The occupancy of activator binding sites is denoted by $f(A)$. The DNA-bound activator recruits the polymerase with an apparent dissociation constant K_A. The DNA-bound repressor competes with the activator for the polymerase. The relevant dissociation constant and the occupancy of the repressor are lumped into $f_1(R)$. The transcription rate is proportional to the occupancy of the promoter by the polymerase recruited by the activator (initiation complex):

$$T \propto \frac{f(A)}{K_A(1+f_1(R))+f(A)} \qquad (1)$$

Supercompetitive inhibition

Elongation or a later step in transcriptional initiation is limited by the permissiveness of the promoter region. The transition between the permissive, P, and non-permissive, N, states is catalyzed by mediators of the activator and repressor, resulting in transition rates ν and π. The proportion of the permissive state is then given by:

$$P = \frac{\pi}{\pi+\upsilon} = \frac{\alpha+f(A)}{\alpha+f(A)+f_2(R)} \qquad (2)$$

where α denotes the basal transition rate to the permissive state. $f_2(R)$ is a compound function that

is determined by the occupancy of the repressor-binding site and the subsequent recruitment of factors that promote the transition from the permissive to the non-permissive state. When $f_2(R)=0$, the state is fully permissive.

Compound repression

The co-occurrence of the initiation complex and the permissive state at a given gene leads to productive transcription. Thus, the probabilities of the two events are multiplied to obtain the transcription rate, with a proportionality constant, w:

$$T = w\frac{f(A)}{K_A(1+f_1(R))+f(A)} * \frac{\alpha+f(A)}{\alpha+f_2(R)+f(A)} \quad (3)$$

Fold inhibition as a function of normalized expression

Fold inhibition (inhibition function) is the ratio of the unrepressed to the repressed transcription rates:

$$i = \frac{K_A(1+f_1(R))+f(A)}{K_A+f(A)} * \frac{\alpha+f_2(R)+f(A)}{\alpha+f(A)} \quad (4)$$

where $f(A)$ is a Hill function, with estradiol concentration e, and the apparent estradiol GEV dissociation constant, K_E:

$$f(A) = \frac{e^n}{K_E^n+e^n}$$

where i can be expressed as a function of estradiol concentration, or normalized unrepressed expression, ne:

$$ne = \frac{E}{E_{max}} = \frac{w\dfrac{f(A)}{K_A+f(A)}}{w\dfrac{1}{K_A}+1} = \frac{f(A)(K_A+1)}{K_A+f(A)}$$

Expressing $f(A)$ in terms of normalized expression and substituting it into Eq. (4) yields the fold inhibition as a function of ne:

$$i = \frac{N1*N2}{(K_A ne+\alpha(1+K_A-ne))(1+K_A)},$$

where

$$\begin{aligned} N1 &= (1+K_A+f_1(R)+K_A f_1(R)-ne\cdot f_1(R)) \\ N2 &= (\alpha(1+K_A-ne)+f_2(R))-ne\cdot f_2(R) \\ &\quad + K_A(ne+f_2(R)) \end{aligned} \quad (5)$$

Fitting of the parameters of the inhibition function

The values of $f_1(R)$ and $f_2(R)$ were fit to individual experiments, while the following parameters were kept fixed: $K_A=1.43$; and $\alpha=0.011$ in Eq. (5). The correlation between $f_1(R)$ and $f_2(R)$ was often higher than 0.99 during nonlinear regression, when supercompetitive inhibition had a larger contribution. In this case, $f_1(R)$ was fixed at a value obtained at the lowest concentration of doxycycline when the value of $f_2(R)$ was still small.

Inhibition functions were fit for the upstream and downstream constructs. The mean inhibition (repression or silencing) strength, I, is defined as the definite integral of the inhibition functions calculated on the interval $ne=[0.06, 0.6]$ to represent the regulatory range around the half-maximal expression.

The amplification factor, a, was fit so that the i_{DUAL} matches the data obtained for the dual repression constructs, assuming:

$$i_{DUAL}-1 = a(i_{US}\cdot i_{DS}-1)$$

where i_{US} and i_{DS} are the inhibition functions of the upstream and downstream repression constructs, respectively. The contribution of supercompetitive inhibition was lower for the upstream silencing than for the downstream silencing construct. Therefore, a was fit using:

$$i_{DUAL}-1 = (a(i_{US}-1)+1)i_{DS}-1$$

for the silencing constructs.

Materials and Methods

Yeast strains and growth conditions

All strains are cogenic with the S288C derivatives, BY4741 (MAT A his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; *gal4::kanMX*) and BY4742 (MAT α his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; *gal4::kanMX*). tetR-Ssn6 is obtained from pCM242.[63] Integration of genetic constructs into various chromosomal loci and copy numbers was verified by Southern blot. All reporter constructs used for silencing have a single-copy integration to avoid long-range interactions. For repression, multiple copy constructs were also used to broaden the range of detectable expression levels. Cells were grown for 6 h after induction in minimal medium supplemented with 2% (w/v) glucose, starting at a cell density of $A_{660}=0.05$. Yeast strains and construction of plasmids with the reporter genes are described in Supplementary Data Tables S3–S6. Measurements of expression at the time points when steady-state expression level is reached are shown in Supplementary Data Fig. S5.

Southern blotting

Yeast genomic DNA was digested with restriction endonucleases and transferred to Hybond-N+ membrane (Amersham) after electrophoresis. DIG-labeled DNA probes were generated with DIG-High Prime (Roche) according to the random primed labeling technique. Anti-digoxigenin alkaline phosphatase was used for detection and the chemiluminescent signal was recorded with a CCD camera (Supplementary Data Fig. S6).

Quantification of RNA levels

Total RNA was isolated by RiboPure Yeast Kit (Ambion). cDNA synthesis was primed with a mix of oligo(dT) and random primers using QuantiTect (Qiagen) and was

quantified by real-time quantitative PCR. *ACT1* was used as an internal standard.

β-Galactosidase assay

Cells were broken in liquid nitrogen by repeated freeze/thaw cycles. The β-galactosidase activity was measured by colorimetry using chlorophenolred-β-D-galactopyranoside as a substrate.

Flow cytometry

GFP expression was measured with flow cytometry using a gating in the side *versus* forward scatter plots to select 5–15% of the total cell population. Gene expression is proportional to the GFP fluorescence:

$$Exp = (F - C)/C$$

F is the fluorescence of cells expressing GFP at the applied concentration of estradiol (e) and doxycycline (d), and C is the background fluorescence when $d=0$, $e=0$. The normalized expression is F/F_{max}, where Exp_{max} is the expression at $e=200$ nM, $d=2$ μM. Fold inhibition − 1 is identical with percentage inhibition divided by 100. When inhibition is weak, the characteristic changes are better displayed when 1 is subtracted from the fold inhibition.

The coefficient of variation of a flow cytometric measurement is less than 5%. When replicates were performed on consecutive days, the error bars representing the standard deviation were typically smaller than the symbols. Larger variations occur when experiments are compared over longer time intervals, in part due to the changes in the estradiol and doxycycline activities in the stock solutions. Since replicates were independent, the parameters were not fit to averages of the replicates but to individual points during nonlinear regression.[64] The mean and standard deviation of the parameters (inhibition strength, amplification factor) were calculated from experiments performed on three different days.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2009.02.025

References

1. Fisher, A. G. (2002). Cellular identity and lineage choice. *Nat. Rev., Immunol.* **2**, 977–982.
2. Glauche, I., Cross, M., Loeffler, M. & Roeder, I. (2007). Lineage specification of hematopoietic stem cells: mathematical modeling and biological implications. *Stem Cells*, **25**, 1791–1799.
3. Komili, S. & Silver, P. A. (2008). Coupling and coordination in gene expression processes: a systems biology view. *Nat. Rev., Genet.* **9**, 38–48.
4. Higgs, D. R., Vernimmen, D., Hughes, J. & Gibbons, R. (2007). Using genomics to study how chromatin influences gene expression. *Annu. Rev. Genomics Hum. Genet.* **8**, 299–325.
5. Murphy, K. F., Balazsi, G. & Collins, J. J. (2007). Combinatorial promoter design for engineering noisy gene expression. *Proc. Natl Acad. Sci. USA*, **104**, 12726–12731.
6. Mikkelsen, T. S., Hanna, J., Zhang, X., Ku, M., Wernig, M., Schorderet, P. *et al.* (2008). Dissecting direct reprogramming through integrative genomic analysis. *Nature*, **454**, 49–55.
7. Green, S. R. & Johnson, A. D. (2004). Promoter-dependent roles for the Srb10 cyclin-dependent kinase and the Hda1 deacetylase in Tup1-mediated repression in Saccharomyces cerevisiae. *Mol. Biol. Cell*, **15**, 4191–4202.
8. Keleher, C. A., Redd, M. J., Schultz, J., Carlson, M. & Johnson, A. D. (1992). Ssn6-Tup1 is a general repressor of transcription in yeast. *Cell*, **68**, 709–719.
9. Smith, R. L. & Johnson, A. D. (2000). Turning genes off by Ssn6-Tup1: a conserved system of transcriptional repression in eukaryotes. *Trends Biochem. Sci.* **25**, 325–330.
10. Klinkenberg, L. G., Mennella, T. A., Luetkenhaus, K. & Zitomer, R. S. (2005). Combinatorial repression of the hypoxic genes of Saccharomyces cerevisiae by DNA binding proteins Rox1 and Mot3. *Eukaryot. Cell*, **4**, 649–660.
11. Malave, T. M. & Dent, S. Y. (2006). Transcriptional repression by Tup1-Ssn6. *Biochem. Cell. Biol.* **84**, 437–443.
12. Fleming, A. B. & Pennings, S. (2007). Tup1-Ssn6 and Swi-Snf remodelling activities influence long-range chromatin organization upstream of the yeast SUC2 gene. *Nucleic Acids Res.* **35**, 5520–5531.
13. Kim, S. J., Swanson, M. J., Qiu, H., Govind, C. K. & Hinnebusch, A. G. (2005). Activator Gcn4p and Cyc8p/Tup1p are interdependent for promoter occupancy at ARG1 in vivo. *Mol. Cell. Biol.* **25**, 11171–11183.
14. Brand, A. H., Breeden, L., Abraham, J., Sternglanz, R. & Nasmyth, K. (1985). Characterization of a "silencer" in yeast: a DNA sequence with properties opposite to those of a transcriptional enhancer. *Cell*, **41**, 41–48.
15. Rusche, L. N., Kirchmaier, A. L. & Rine, J. (2003). The establishment, inheritance, and function of silenced chromatin in Saccharomyces cerevisiae. *Annu. Rev. Biochem.* **72**, 481–516.
16. Moazed, D. (2001). Common themes in mechanisms of gene silencing. *Mol. Cell*, **8**, 489–498.
17. Gao, L. & Gross, D. S. (2008). Sir2 silences gene transcription by targeting the transition between RNA polymerase II initiation and elongation. *Mol. Cell. Biol.* **28**, 3979–3994.
18. Chen, L. & Widom, J. (2005). Mechanism of transcriptional silencing in yeast. *Cell*, **120**, 37–48.
19. Gotta, M., Strahl-Bolsinger, S., Renauld, H., Laroche, T., Kennedy, B. K., Grunstein, M. & Gasser, S. M. (1997). Localization of Sir2p: the nucleolus as a compartment for silent information regulators. *EMBO J.* **16**, 3243–3255.
20. Meneghini, M. D., Wu, M. & Madhani, H. D.

(2003). Conserved histone variant H2A.Z protects euchromatin from the ectopic spread of silent heterochromatin. *Cell*, **112**, 725–736.
21. King, D. A., Hall, B. E., Iwamoto, M. A., Win, K. Z., Chang, J. F. & Ellenberger, T. (2006). Domain structure and protein interactions of the silent information regulator Sir3 revealed by screening a nested deletion library of protein fragments. *J. Biol. Chem.* **281**, 20107–20119.
22. Yang, B. & Kirchmaier, A. L. (2006). Bypassing the catalytic activity of SIR2 for SIR protein spreading in Saccharomyces cerevisiae. *Mol. Biol. Cell*, **17**, 5287–5297.
23. Rusche, L. N., Kirchmaier, A. L. & Rine, J. (2002). Ordered nucleation and spreading of silenced chromatin in Saccharomyces cerevisiae. *Mol. Biol. Cell*, **13**, 2207–2222.
24. Yang, B., Miller, A. & Kirchmaier, A. L. (2008). HST3/HST4-dependent deacetylation of lysine 56 of histone H3 in silent chromatin. *Mol. Biol. Cell*, **19**, 4993–5005.
25. Talbert, P. B. & Henikoff, S. (2006). Spreading of silent chromatin: inaction at a distance. *Nat. Rev., Genet.* **7**, 793–803.
26. Dodd, I. B., Shearwin, K. E., Perkins, A. J., Burr, T., Hochschild, A. & Egan, J. B. (2004). Cooperativity in long-range gene regulation by the lambda CI repressor. *Genes Dev.* **18**, 344–354.
27. Kuhlman, T., Zhang, Z., Saier, M. H., Jr & Hwa, T. (2007). Combinatorial transcriptional control of the lactose operon of Escherichia coli. *Proc. Natl Acad. Sci. USA*, **104**, 6043–6048.
28. Gardner, T. S., Cantor, C. R. & Collins, J. J. (2000). Construction of a genetic toggle switch in Escherichia coli. *Nature*, **403**, 339–342.
29. Muller, J., Barker, A., Oehler, S. & Muller-Hill, B. (1998). Dimeric lac repressors exhibit phase-dependent co-operativity. *J. Mol. Biol.* **284**, 851–857.
30. Bintu, L., Buchler, N. E., Garcia, H. G., Gerland, U., Hwa, T., Kondev, J. & Phillips, R. (2005). Transcriptional regulation by the numbers: models. *Curr. Opin. Genet. Dev.* **15**, 116–124.
31. Ozbudak, E. M., Thattai, M., Lim, H. N., Shraiman, B. I. & Van Oudenaarden, A. (2004). Multistability in the lactose utilization network of Escherichia coli. *Nature*, **427**, 737–740.
32. Frolova, E., Johnston, M. & Majors, J. (1999). Binding of the glucose-dependent Mig1p repressor to the GAL1 and GAL4 promoters in vivo: regulationby glucose and chromatin structure. *Nucleic Acids Res.* **27**, 1350–1358.
33. Gao, C. Y. & Pinkham, J. L. (2000). Tightly regulated, beta-estradiol dose-dependent expression system for yeast. *BioTechniques*, **29**, 1226–1231.
34. Setty, Y., Mayo, A. E., Surette, M. G. & Alon, U. (2003). Detailed map of a cis-regulatory input function. *Proc. Natl Acad. Sci. USA*, **100**, 7702–7707.
35. Zaman, Z., Heid, C. & Ptashne, M. (2002). Telomere looping permits repression "at a distance" in yeast. *Curr. Biol.* **12**, 930–933.
36. Rusche, L. N. & Rine, J. (2001). Conversion of a gene-specific repressor to a regional silencer. *Genes Dev.* **15**, 955–967.
37. Guo, Z. & Sherman, F. (1995). 3'-end-forming signals of yeast mRNA. *Mol. Cell Biol.* **15**, 5983–5990.
38. Cornish-Bowden, A. (2004). *Fundamentals of Enzyme Kinetics*, 3rd edit. Portland Press, London.
39. Dhasarathy, A. & Kladde, M. P. (2005). Promoter occupancy is a major determinant of chromatin remodeling enzyme requirements. *Mol. Cell. Biol.* **25**, 2698–2707.
40. Hecht, A., Strahl-Bolsinger, S. & Grunstein, M. (1996). Spreading of transcriptional repressor SIR3 from telomeric heterochromatin. *Nature*, **383**, 92–96.
41. Chou, C. C., Li, Y. C. & Gartenberg, M. R. (2008). Bypassing Sir2 and O-acetyl-ADP-ribose in transcriptional silencing. *Mol. Cell*, **31**, 650–659.
42. O'Sullivan, J. M., Tan-Wong, S. M., Morillon, A., Lee, B., Coles, J., Mellor, J. & Proudfoot, N. J. (2004). Gene loops juxtapose promoters and terminators in yeast. *Nat. Genet.* **36**, 1014–1018.
43. Renauld, H., Aparicio, O. M., Zierath, P. D., Billington, B. L., Chhablani, S. K. & Gottschling, D. E. (1993). Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by SIR3 dosage. *Genes Dev.* **7**, 1133–1145.
44. Boscheron, C., Maillet, L., Marcand, S., Tsai-Pflugfelder, M., Gasser, S. M. & Gilson, E. (1996). Cooperation at a distance between silencers and proto-silencers at the yeast HML locus. *EMBO J.* **15**, 2184–2195.
45. Pillus, L. & Rine, J. (2004). SIR1 and the origin of epigenetic states in Saccharomyces cerevisiae. *Cold Spring Harbor Symp. Quant. Biol.* **69**, 259–265.
46. Xu, E. Y., Zawadzki, K. A. & Broach, J. R. (2006). Single-cell observations reveal intermediate transcriptional silencing states. *Mol. Cell*, **23**, 219–229.
47. Fourel, G., Revardel, E., Koering, C. E. & Gilson, E. (1999). Cohabitation of insulators and silencing elements in yeast subtelomeric regions. *EMBO J.* **18**, 2522–2537.
48. Pryde, F. E. & Louis, E. J. (1999). Limitations of silencing at native yeast telomeres. *EMBO J.* **18**, 2538–2550.
49. Robyr, D., Suka, Y., Xenarios, I., Kurdistani, S. K., Wang, A., Suka, N. & Grunstein, M. (2002). Microarray deacetylation maps determine genome-wide functions for yeast histone deacetylases. *Cell*, **109**, 437–446.
50. DeRisi, J. L., Iyer, V. R. & Brown, P. O. (1997). Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science*, **278**, 680–686.
51. Kuchin, S. & Carlson, M. (1998). Functional relationships of Srb10-Srb11 kinase, carboxy-terminal domain kinase CTDK-I, and transcriptional corepressor Ssn6-Tup1. *Mol. Cell. Biol.* **18**, 1163–1171.
52. Zhang, Z. & Reese, J. C. (2004). Redundant mechanisms are used by Ssn6-Tup1 in repressing chromosomal gene transcription in Saccharomyces cerevisiae. *J. Biol. Chem.* **279**, 39240–39250.
53. Lee, M., Chatterjee, S. & Struhl, K. (2000). Genetic analysis of the role of Pol II holoenzyme components in repression by the Cyc8-Tup1 corepressor in yeast. *Genetics*, **155**, 1535–1542.
54. Kimura, A., Umehara, T. & Horikoshi, M. (2002). Chromosomal gradient of histone acetylation established by Sas2p and Sir2p functions as a shield against gene silencing. *Nature Genet.* **32**, 370–377.
55. McBryant, S. J., Krause, C., Woodcock, C. L. & Hansen, J. C. (2008). The silent information regulator 3 protein, SIR3p, binds to chromatin fibers and assembles a hypercondensed chromatin architecture in the presence of salt. *Mol. Cell. Biol.* **28**, 3563–3572.
56. Andrau, J. C., van de Pasch, L., Lijnzaad, P., Bijma, T., Koerkamp, M. G., van de Peppel, J. *et al.* (2006). Genome-wide location of the coactivator mediator: Binding without activation and transient Cdk8 interaction on DNA. *Mol. Cell*, **22**, 179–192.

57. Zhu, X., Wiren, M., Sinha, I., Rasmussen, N. N., Linder, T., Holmberg, S. *et al.* (2006). Genome-wide occupancy profile of mediator and the Srb8–11 module reveals interactions with coding regions. *Mol. Cell*, **22**, 169–178.
58. Batada, N. N., Urrutia, A. O. & Hurst, L. D. (2007). Chromatin remodelling is a major source of coexpression of linked genes in yeast. *Trends Genet.* **23**, 480–484.
59. Hurst, L. D., Williams, E. J. & Pal, C. (2002). Natural selection promotes the conservation of linkage of co-expressed genes. *Trends Genet.* **18**, 604–606.
60. Becskei, A., Kaufmann, B. B. & van Oudenaarden, A. (2005). Contributions of low molecule number and chromosomal positioning to stochastic gene expression. *Nat. Genet.* **37**, 937–944.
61. Safi, A., Wallace, K. A. & Rusche, L. N. (2008). Evolution of new function through a single amino acid change in the yeast repressor Sum1p. *Mol. Cell. Biol.* **28**, 2567–2578.
62. Becskei, A. & Grusby, M. J. (2007). Contribution of IL-12R mediated feedback loop to Th1 cell differentiation. *FEBS Lett.* **581**, 5199–5206.
63. Belli, G., Gari, E., Piedrafita, L., Aldea, M. & Herrero, E. (1998). An activator/repressor dual system allows tight tetracycline-regulated gene expression in budding yeast. *Nucleic Acids Res.* **26**, 942–947.
64. Motulsky, H. & Christopoulos, A. (2004). *Fitting Models to Biological Data Using Linear and Nonlinear Regression*. Oxford University Press, New York.

Supplementary Information

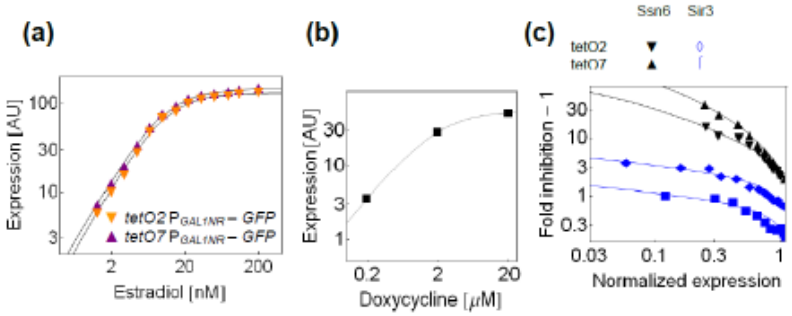

Fig. S1. Regulation of gene expression by GEV and rtTA; tetR-Ssn6 and tetR-Sir3, when they bind to two or seven tet operators upstream of the reporter gene.
(a) Expression in non-repressive conditions (doxycycline = 2 μM) are shown for the tetO2-P_{GAL1NR} and tetO7-P_{GAL1NR} genetic constructs (PRY377, 449). The induction curves are nearly identical.
(b) Expression induced by tetR'-VP16 (rtTA) at tetO7-P_{GAL1NR} construct (PRY369). rtTA is expressed is the same way is in Fig. 1d; thus, it serves as a positive control.
(c) Dependence of the inhibition function on the number of operators.
The contribution of supercompetitive repression increased by a factor of 2.6 when tetR-Ssn6 binds to seven instead of two operators (PRY390, 454, $f_1(R)$ was kept equal for the two strains). The mean silencing strength increased from 0.85 to 2.5 when tetR-Sir3 binds to seven instead of two operators (PRY378, 370).

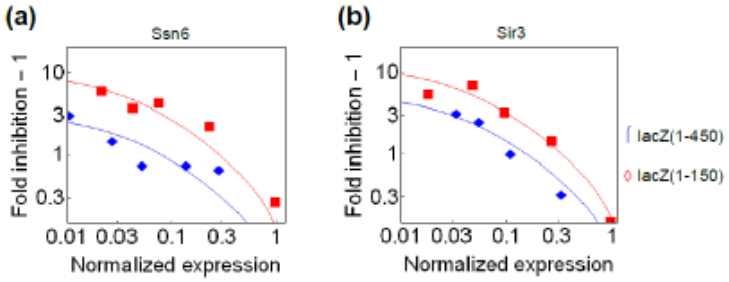

Fig. S2. Downstream repression and silencing of short reporter constructs.
(A and B) Expression of 0.15 and 0.45 kb fragments of lacZ was quantified by real-time PCR. The repression strengths of the respective constructs (PRY490.1, 493.1) were 1.13 and 0.36 (A) and the silencing strengths (PRY491.1, PRY494.1) were 1.36 and 0.62 (B).

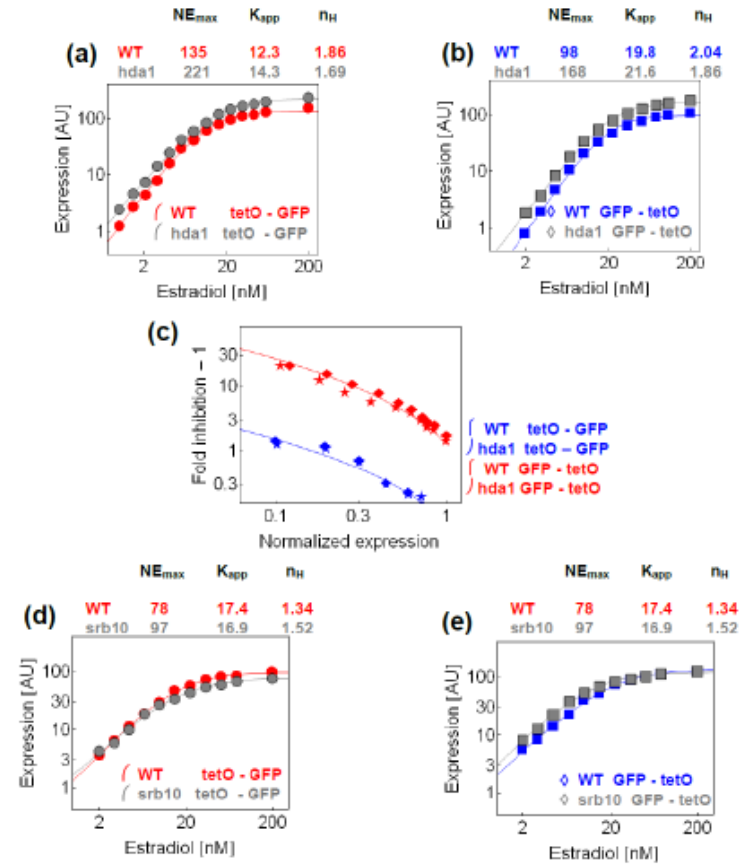

Fig. S3. Effect of disruption of *HDA1* and *SRB1* on gene expression
Parameters of the fitted Hill function for data shown above the panels.

$$Expression = \frac{est^n}{est^n + K_{app}^n}$$

(a and b) Induction curves under non-repressive conditions. The maximum expression increases approximately 70% in hda1 (PRY401, 415) cells relative to wild-type cells (PRY393, 418)

(c) *HDA1* disruption increases the absolute expression rate but repression strength is unaffected. The mean relative difference of repression strength between the hda1 and WT cells are -0.01 ± 0.18 and 0.06 ± 0.25 for tetO – GFP and GFP – tetO constructs, respectively. Thus, the change is less than 10%.

(d and e) Induction curves for wild-type and srb10 cells. Expression in non-repressive condition is shown for PRY393, 441 (D) and PRY418, 445 (E).

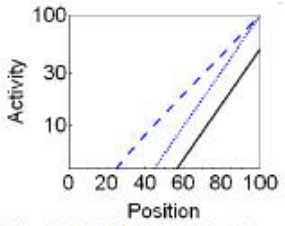

Fig. S4. Facilitation of gradients.
Only nucleation (blue dotted) or spreading and nucleation (blue dashed line) are enhanced relative to the primary gradient (black full line).
Steady state solution of the reaction-diffusion equation $0.3*D^2_x\ a(x)-k*a(x) = 0$ is shown with the following boundary conditions: $a(0) = 0$; $a(100) = 50$ and 100 for the black and blue lines, respectively. a denotes the activity (concentration) of the compound, which diffuses along the chromosome in 1 dimension. The rate of dissociation from the chromosome, k, is 0.0005 (dashed lines) or 0.001 (dotted lines).

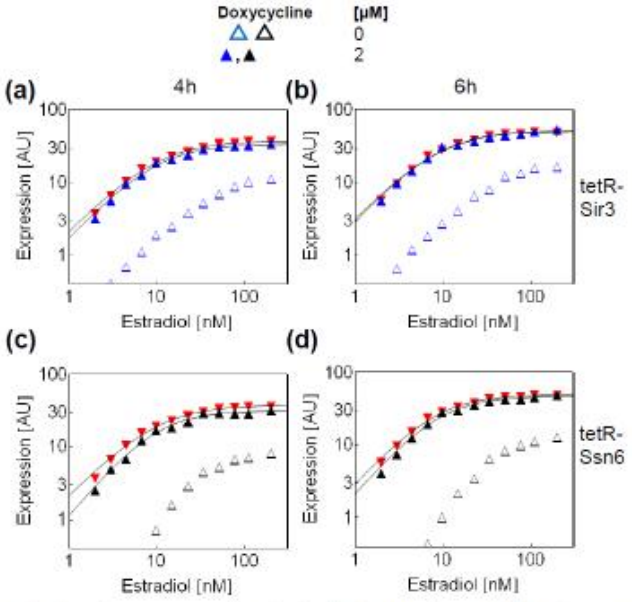

Fig. S5. Kinetics of gene expression in the dual repression and silencing constructs. There is a minor change in the induction curves at 6 hours after induction relative to

the 4 hour induction curves. Thus, 6 hour induction curves were considered to represent steady-state expression levels. Red triangles represent strains containing GEV only, while blue and black triangles represent strains that contain tetR-Sir3 or tetR-Ssn6 in addition to GEV. (A and B) PRY354, 355 (C and D) PRY354, 355.1

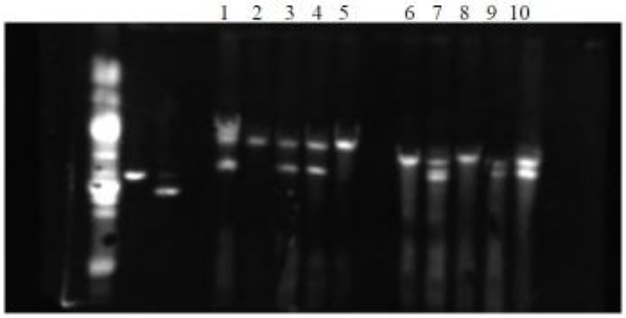

Figure S6. Southern-blot of genomic DNA containing the reporter constructs.
Genomic DNA from YSSH162.1, 2, 4, 5, 6 (1-5) cells was digested with BamHI, while the genomic DNA from YSSH161.2, 3, 4, 5, 6 (6-10) cells was digested with BamHI and MluI. Single bands indicate a single copy, while two bands indicate 2 or more copies of the reporter construct integrated into the *YFR054C* and the *BAT2* loci, respectively.

	0	**200**	Estradiol [nM]
Consruct	**0**	**2**	Doxycycline [μM]
P_{GALINR} - GFP-T_{ACT1}-lacZ-$[tetO]_4$	2.1	2.4	β-Galactosidase units
P_{GALINR} - GFP-T_{ACT1}-lacZ-$[tetO]_4$	<0.5	52	Fluorescence [AU]
P_{GALINR} - lacZ-$[tetO]_4$	0.9	416	β-Galactosidase units

Table S1. β-Galactosidase activity of the GFP-T-lacZ construct. A lacZ-tetO4 construct is included, as a positive control.

α-factor [nM]	Doxycycline [μM]	*FIG1* RNA tetR-Ssn6	*FIG1* RNA No repressor	Fold-change dox (+/-) tetR-Ssn6	Fold-change dox (+/-) No repressor
30	**0**	40 ± 13	241 ± 20	3.83	1.92
30	**2**	155 ± 3	463 ± 11		
300	**0**	210 ± 6	2215 ±120	5.02	2.1
300	**2**	1055 ± 42	4644 ± 780		

Table S2. Effect of TetR-Ssn6 binding downstream of *FIG1* on *FIG1* expression..
Overnight cultures were diluted to OD_{600} = 0.15 and grown for 90 minutes (in the presence of doxycycline as indicated). α-factor was then added and cells were harvested 60 minutes after induction of *FIG1* expression with α-factor. A non-specific effect of doxycycline on *FIG1* expression was observed in strains that do not have tetR-Ssn6. Thus, the normalized repression factor of *FIG1* expression is approximately 2.
Strains carrying tetR-Ssn6 have an approximately 4fold reduction of *FIG1* expression in the presence of doxycycline (nonrepressive condition). This could be due to the overexpression of Ssn6, since some of the mating factor regulated genes are affected by Ssn6.

Table S3. Plasmid list

Plasmid Number	Description	Construction
pPR1	pRS303::P_{MRP7}-GEV-T_{ACT1}	P_{MRP7}-GEV Insert described in Gao & Pinkham, 2000: ApaI-P_{MRP7}-SgsI-GEV-EcoRI-T_{ACT1}-NotI
pPR2	pRS306::*FIG1*(Int)-tetO7-P_{GAL1NR}-GFP-T_{CYC1}	KpnI-FIG1-XhoI-T_{ADH1}-tetO7-SphI-P_{GAL1NR}-BamHI-GFP-EcoRI-T_{CYC1}-NotI
pPR4	pRS306::P_{RET2}(Int)-tetR-SSN6-T_{ACT1}	KpnI-P_{RET2}-XbaI-tetR-SSN6-EcoRI-SpeI-Tact1-NotI
pPR6	pRS306::*FIG1*(Int)-tetO7-P_{GAL1NR}-GFP-T_{CYC1}-E-element	KpnI-FIG1-XhoI-T_{ADH1}-tetO7-SphI-P_{GAL1NR}-BamHI-GFP-EcoRI-T_{CYC1}-XbaI-E-element-NotI
pPR8	pRS306::*FIG1*(Int)-T_{ADH1}-tetO2-P_{GAL1NR}-GFP-T_{CYC1}	KpnI-FIG1-XhoI-T_{ADH1}-tetO2-SphI-P_{GAL1NR}-BamHI-GFP-EcoRI-T_{CYC1}-NotI
pPR9	pRS306::*FIG1*(Int)-T_{ADH1}-tetO2-P_{GAL1NR}-GFP-tetO4	KpnI-FIG1-XhoI-T_{ADH1}-tetO2-SphI-P_{GAL1NR}-BamHI-GFP-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR10	pRS306::*FIG1*(Int)-T_{GAL7}-P_{GAL1NR}-GFP-tetO4	KpnI-FIG1-XhoI-T_{GAL7}-SphI-P_{GAL1NR}-BamHI-GFP-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR11	pRS306::*FIG1*(Int)-tetO7-P_{GAL1NR}-GFP-tetO4	KpnI-FIG1-XhoI-T_{ADH1}-tetO7-SphI-P_{GAL1NR}-BamHI-GFP-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR13	pRS306::P_{RET2}(Int)-tetR-SIR3-T_{ACT1}	KpnI-P_{RET2}-XbaI-tetR-HindIII-BamHI-SIR3-SpeI-Tact1-NotI
pPR17	pRS306::*FIG1*(Int)-tetO7-P_{GAL1NR}-[GFP]2-tetO4	KpnI-FIG1-XhoI-T_{ADH1}-tetO7-SphI-P_{GAL1NR}-BamHI-GFP-BsrGI/Acc65I-GFP-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR33	pRS306::*FIG1*(Int)-T_{ADH1}-tetO2-P_{GAL1NR}-[GFP]2-tetO4	KpnI-FIG1-XhoI-T_{ADH1}-tetO2-SphI-P_{GAL1NR}-BamHI-GFP-BsrGI/Acc65I-GFP-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR34	pRS306::*FIG1*(Int)-T_{ADH1}-tetO2-P_{GAL1NR}-[GFP]2-T_{CYC1}	KpnI-FIG1-XhoI-T_{ADH1}-tetO2-SphI-P_{GAL1NR}-BamHI-GFP-BsrGI/Acc65I-GFP-EcoRI-T_{CYC1}-NotI
pPR35	pRS306::*FIG1*(Int)-T_{ADH1}-tetO2-P_{GAL1NR}-GFP-T_{ACT1}-lacZ-T_{CYC1}	KpnI-FIG1-XhoI-T_{ADH1}-tetO2-SphI-P_{GAL1NR}-BamHI-GFP-SpeI-TACT1-BglII/BamHI-lacZ-EcoRI-T_{CYC1}-NotI
pPR36	pRS306::*FIG1*(Int)-T_{ADH1}-tetO2-P_{GAL1NR}-GFP-T_{ACT1}-YFP-tetO4	KpnI-FIG1-XhoI-T_{ADH1}-tetO2-SphI-P_{GAL1NR}-BamHI-GFP-SpeI-TACT1-BglII/BamHI-YFP-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR37	pRS306::*FIG1*(Int)-T_{ADH1}-tetO2-P_{GAL1NR}-GFP-T_{ACT1}-lacZ-tetO4	KpnI-FIG1-XhoI-T_{ADH1}-tetO2-SphI-P_{GAL1NR}-BamHI-GFP-SpeI-TACT1-BglII/BamHI-lacZ-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR40	pRS306::*YFR054C*(Int)-tetO7-P_{GAL1NR}-GFP-tetO4	KpnI-YFR054C-SalI/XhoI-T_{ADH1}-tetO7-SphI-P_{GAL1NR}-BamHI-GFP-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI

pPR46	pRS306::*DAN1*(Int)-T_{GAL7}-P_{GAL1NR}-GFP-tetO4	KpnI-DAN1-XhoI-T_{GAL7}-SphI-P_{GAL1NR}- BamHI-GFP-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR48	pRS306::*DAN1*(Int)-T_{GAL7}-P_{GAL1NR}-GFP-T_{ACT1}-YFP-tetO4	KpnI-DAN1-XhoI-T_{GAL7}-SphI-P_{GAL1NR}-BamHI-GFP-SpeI-TACT1-BglII/BamHI-YFP-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR54	pRS306::*BAT2*(Int)-T_{GAL7}-P_{GAL1NR}-GFP-tetO4	KpnI-BAT2-XhoI-T_{GAL7}-SphI-P_{GAL1NR}-BamHI-GFP-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR61	pRS306::*YFR054C*(Int)-tetO7-P_{GAL1NR}-GFP-T_{CYC1}	KpnI-YFR054C-SalI/XhoI-T_{ADH1}-tetO7-SphI-P_{GAL1NR}-BamHI-GFP-EcoRI-T_{CYC1}-NotI
pPR67	pRS306::*YFR054C+ter*(Int)-tetO2-Spacer B-T_{GAL7}-P_{GAL1NR}-GFP-T_{CYC1}	KpnI-YFR054C+ter-SpeI-tetO2-SgsI-Spacer B-SalI/XhoI-T_{GAL7}-P_{GAL1NR}-BamHI-GFP-EcoRI-T_{CYC1}-NotI
pPR70	pRS306::*YFR054C*(Int)-T_{ADH1}-tetO2 -P_{GAL1NR}-GFP-T_{CYC1}	KpnI-YFR054C-SalI/XhoI-T_{ADH1}-tetO2-SphI-P_{GAL1NR}-BamHI-GFP-EcoRI-T_{CYC1}-NotI
pPR71	pRS306::*YFR054C*(Int)-T_{GAL7}-P_{GAL1NR}-GFP-tetO4	KpnI-YFR054C-SalI/XhoI-T_{GAL7}-SphI-P_{GAL1NR}-BamHI-GFP-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR74	pRS306::*YFR054C*(Int)-T_{ADH1}-tetO2 -P_{GAL1NR}-GFP-tetO4	KpnI-YFR054C-SalI/XhoI-T_{ADH1}-tetO2-SphI-P_{GAL1NR}-BamHI-GFP-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR78	pRS306::*YFR054C*(Int)-T_{ADH1}-tetO2 -P_{GAL1NR}-GFP-T_{ACT1}-YFP-tetO4	KpnI-YFR054C-SalI/XhoI-T_{ADH1}-tetO2-SphI-P_{GAL1NR}-BamHI-GFP-SpeI-TACT1-BglII/ BamHI-YFP-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR79	pRS306::*YFR054C*(Int)-tetO7-P_{GAL1NR}-GFP-T_{ACT1}-lacZ-tetO4	KpnI-YFR054C-SalI/XhoI-T_{ADH1}-tetO7-SphI-P_{GAL1NR}-BamHI-GFP-SpeI-TACT1-BglII/ BamHI-lacZ-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR80	pRS306::*YFR054C*(Int)-T_{ADH1}-tetO2 -P_{GAL1NR}-GFP-T_{ACT1}-lacZ-tetO4	KpnI-YFR054C-SalI/XhoI-T_{ADH1}-tetO2-SphI-P_{GAL1NR}-BamHI-GFP-SpeI-TACT1-BglII/ BamHI-lacZ-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR81	pRS306::*YFR054C*(Int)-T_{GAL7}-P_{GAL1NR}-GFP-T_{ACT1}-YFP-tetO4	KpnI-YFR054C-SalI/XhoI-T_{GAL7}-SphI-P_{GAL1NR}-BamHI-GFP-SpeI-TACT1-BglII/BamHI-YFP-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR82	pRS306::*YFR054C*(Int)-T_{GAL7}-P_{GAL1NR}-[GFP]2-tetO4	KpnI-YFR054C-SalI/XhoI-T_{GAL7}-SphI-P_{GAL1NR}-BamHI-GFP-BsrGI/Acc65I-GFP-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR83	pRS306::*YFR054C*(Int)-T_{GAL7}-P_{GAL1NR}-GFP-T_{ACT1}-lacZ-tetO4	KpnI-YFR054C-SalI/XhoI-T_{GAL7}-SphI-P_{GAL1NR}-BamHI-GFP-SpeI-TACT1-BglII/BamHI-lacZ-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR88	pRS306::*YFR054C+ter*(Int)-tetO2-Spacer B-T_{GAL7}-P_{GAL1NR}-GFP-TetO4	KpnI-YFR054C+ter-SpeI-tetO2-SgsI-Spacer B-SalI/XhoI-T_{GAL7}-P_{GAL1NR}-BamHI-GFP-EcoRI-T_{ADH1}-TetO4-SphI-T_{ACT1}-NotI
pPR89	pRS305::P_{RET2}(Int)-tetR-SIR3-T_{ACT1}	KpnI-P_{RET2}-XbaI-tetR-HindIII-BamHI-SIR3-SpeI-Tact1-NotI

pPR90	pRS305::P_{RET2}(Int)-tetR-SSN6-T_{ACT1}	KpnI-P_{RET2}-XbaI-tetR-SSN6-EcoRI-SpeI-Tact1-NotI
pPR92	pRS306::*DAN1*(Int)-T_{GAL7}-P_{GAL1NR}-GFP-T_{ACT1}-lacZ-tetO4	KpnI-DAN1-XhoI-T_{GAL7}-SphI-P_{GAL1NR}-BamHI-GFP-SpeI-TACT1-BglII/BamHI-lacZ-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR93	pRS305::*HDA1*	HindIII-HDA1 (+303 to +885)-SpeI
pPR100	pRS306::*DAN1*(Int)-T_{GAL7}-$P_{GAL1(Mig1)}$-GFP-T_{ACT1}-lacZ-tetO4	KpnI-DAN1-XhoI-T_{GAL7}-SphI-P_{GAL1}-BamHI-GFP-SpeI-TACT1-BglII/BamHI-lacZ-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR101	pRS305::*SRB10*	XhoI-SRB10 (+10 to +417)-SpeI
pPR105	pRS306::*YFR054C*(Int)-T_{GAL7}-P_{GAL1NR}-lacZ(150)-tetO4	KpnI-YFR054C-SalI/XhoI-T_{GAL7}-SphI-P_{GAL1NR}-BamHI-lacZ(150)-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR106	pRS306::*YFR054C*(Int)-T_{GAL7}-P_{GAL1NR}-lacZ(450)-tetO4	KpnI-YFR054C-SalI/XhoI-T_{GAL7}-SphI-P_{GAL1NR}-BamHI-lacZ(450)-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR109	pRS306::*YFR054C+ter*(Int)-tetO2-Spacer B(300)-T_{GAL7}-P_{GAL1NR}-GFP-T_{CYC1}	KpnI-YFR054C+ter-SpeI-tetO2-SgsI-Spacer B(300)-SalI/XhoI-T_{GAL7}-P_{GAL1NR}-BamHI-GFP-EcoRI- T_{CYC1}-NotI
pPR111	pRS306::*YFR054C+ter*(Int)-tetO2-T_{GAL7}-P_{GAL1NR}-GFP- T_{CYC1}	KpnI-YFR054C+ter-SpeI-tetO2-SgsI-T_{GAL7}-SphI-P_{GAL1NR}-BamHI-GFP-EcoRI-T_{CYC1}-NotI
pPR112	pRS306::*YFR054C+ter*(Int)-tetO2-T_{GAL7}-P_{GAL1NR}-GFP- tetO4	KpnI-YFR054C+ter-SpeI-tetO2-SgsI-T_{GAL7}-SphI-P_{GAL1NR}-BamHI-GFP-EcoRI- T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR115	pRS306::*IRC7*(Int)-T_{GAL7}-P_{GAL1NR}-GFP-tetO4	KpnI-IRC7-XhoI-T_{GAL7}-SphI-P_{GAL1NR}-BamHI-GFP-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR116	pRS306::*YFR054C*(Int)-T_{ADH1}-tetO2-P_{GAL1NR}-[GFP]2-tetO4	KpnI-YFR054C-SalI/XhoI-T_{ADH1}-tetO2-SphI-P_{GAL1NR}-BamHI- GFP-BsrGI/Acc65I-GFP-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pPR126	pRS306::*FIG1*(Int)-T_{ADH1}-tetO7 -P_{GAL1NR}-GFP-T_{ACT1}-lacZ-tetO4	KpnI-FIG1-XhoI-T_{ADH1}-tetO2-SphI-P_{GAL1NR}-BamHI-GFP-SpeI-TACT1-BglII/BamHI-lacZ-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI
pCF101	pRS306::*FIG1*(Int)-T_{GAL7}-P_{GAL1NR}-[GFP]2-tetO4	KpnI-FIG1-XhoI-T_{GAL7}-SphI-P_{GAL1NR}-BamHI-GFP-BsrGI/Acc65I-GFP-EcoRI-T_{ADH1}-tetO4-SphI-T_{ACT1}-NotI

DESCRIPTION OF SEQUENCES

P_{GAL1NR}

SphI-GALUAS-SalI-GAL1TATA-BamHI (without Mig1 binding sites) (NR-no repression)

GCATGCATATTGAAGTACGGATTAGAAGCCGCCGAGCGGGCGACAGCCCT
CCGACGGAAGACTCTCCTCCGTGCGTCCTCGTCTTCACCGGTCGCGTTCCT
GAAACGCAGATGTGCCTCGCGCCGCACTGCTCCGAACAATAAAGATTCTA
CAATACTAGCTTTTATGGTTATGAAGAGGAAAAATTGGCAGTAACCTGGC
CCCACAAACCTTCAAATTAACGAATCAAATTAACAACCATAGGATGATAA
TGCGATTAGTTTTTTAGCCTTATTTCTGGGGTAATTAATCAGCGAAGCGAT
GATTTTTGATCTATTAACAGATATATAAATGGAAAAGCTGCATAACCACTT
TAACTAATACTTTCAACATTTTCAGTTTGTATTACTTCTTATTCAAATGTCA
TAAAAGTATCAACAAAAAATTGTTAATATACCTCTATACTTTAACGTCAAG
GAGAAAAAACTATAGGATCC

P_{MRP7}-GEV (Gal4DNA binding domain, Estradiol receptor, VP16 activator domain)

Promoter Region of MRP7 (-904 to +1) and GEV of pGEVLeu2 (Gao & Pinkham, 2000) were ligated with SgsI and then cloned into pRS303 cut with ApaI and EcoRI.

tetR

Sequence between XbaI and HindIII of pCM217 that binds to tet operator.

tetR-SSN6

Sequence between XbaI and MfeI of pCM242, which encodes the fusion protein tetR - Ssn6p.

tetO4

tetO4 is a jumping product obtained from tetO7 during Polymerase Chain Reaction.

E-element

E-element is HML-E silencer. The DNA sequence corresponds to coordinates 11146 to 11401 on Chromosome III.

GAATCAAATAGGTGTATCGCAATGGAATGTAATTTCTTAAGTATTCTATATGT
ACTTAAAACCTATTAATATATGGATCAACACAGTATCTTATGAATGGGTTTTT
GATTTTTTTATGTTTTTTTAAAACATTAAAGTTTTCGGCACGGACTTATTTGG
AATTCAAATTATTAATGAAAGAACAATTAACTAATTAATGTACTTAGTATTTG
GCCATTATTATCGATTTCGGGGGCCAAATCTAACCAAATTCAAC

ABBREVIATIONS

tetO4	T_{ADH1}-tetO4
tetO7	T_{ADH1}-tetO7
[GFP]2	BamHI-GFP-BsrGI/Acc65I-GFP-EcoRI
GFP-T_{ACT1}-YFP	BamHI-GFP-SpeI-TACT1-BglII/BamHI-YFP-EcoRI
GFP-T_{ACT1}-lacZ	BamHI-GFP-SpeI-TACT1-BglII/BamHI-lacZ-EcoRI
Spacer B	+514 to +1085 of RPN12
P_{RET2}	-710 to +21 of RET2
T_{ADH1}	ADH1 Terminator
T_{ACT1}	ACT1 Terminator
T_{GAL7}	GAL7 Terminator
T_{CYC1}	CYC1 Terminator
P_{MRP7}	-904 to +1 of MRP7
FIG1	+180 to +898 of FIG1
DAN1	+1386 to +551 of DAN1
YFR054C	+1 to +413 of YFR054C
YFR054C+ter	+1 to +987 of YFR054C
BAT2	+1183 to +1632 of BAT2
HDA1	+303 to +885 of HDA1
SRB10	+10 to +417 of SRB10
UBP10	+42 to +526 of UBP10

Table S4. Diploid strain list
The diploid strains were obtained by mating the following haploid strains.

Diploid	**MAT A Haploid**	**MAT α Haploid**
Strain Number	Strain Number	Strain Number
PRY342	PRY264	YSSH211.3
PRY351	YSSH162.2	YSSH211.3
PRY354	YSSH168.4	YSSH208
PRY355	YSSH168.4	YSSH211.3
PRY355.1	YSSH168.4	PRY373
PRY364	YSSH167.3	YSSH211.3
PRY365	YSSH185.1	YSSH211.3
PRY366	YSSH184.2	YSSH211.3
PRY367	YSSH186.1	YSSH211.3
PRY368	PRY263	YSSH215.4
PRY369	PRY002	YSSH215.4
PRY370	PRY002	YSSH211.3
PRY371	PRY033	YSSH211.3
PRY372	PRY043	YSSH211.3
PRY377	YSSH075	PRY373
PRY378	YSSH074	YSSH211.3
PRY379	YSSH077	YSSH211.3
PRY379A	PRY265	PRY373
PRY386	YSSH097	PRY373
PRY387	YSSH167.1	PRY373
PRY389	PRY033	PRY373
PRY390	YSSH074	PRY373
PRY391	YSSH077	PRY373
PRY393	YSSH162.5	PRY373
PRY401	PRY397	PRY396.7
PRY415	YSSH223.1	PRY396.8
PRY418	YSSH167.3	PRY373
PRY419	YSSH185.1	PRY373
PRY420	YSSH184.2	PRY373
PRY421	YSSH186.1	PRY373
PRY422B	YSSH152.3	YSSH211.3
PRY423B	YSSH194.1	YSSH211.3
PRY430	YSSH167.6	PRY373
PRY432	YSSH162.4	PRY373
PRY434	PRY224	YSSH211.3
PRY435	YSSH022	YSSH211.3
PRY436	YSSH040	YSSH211.3
PRY438	YSSH162.2	PRY373
PRY441	PRY467.1	PRY466.1
PRY445	PRY468.2	PRY466.1
PRY449	YSSH70	PRY373
PRY450B	YSSH152.3	PRY373
PRY451B	YSSH194.1	PRY373

PRY453	PRY470.3	YSSH211.3
PRY457	PRY455	PRY373
PRY461	YSSH071	YSSH211.3
PRY462	YSSH043	YSSH211.3
PRY474.4	PRY473.4	YSSH211.3
PRY475.4	PRY473.4	PRY373
PRY478.1	PRY476.1	PRY373
PRY483.1	PRY485.1	PRY373
PRY490.1	PRY489.1	PRY373
PRY491.1	PRY489.1	YSSH211.3
PRY493.1	PRY492.1	PRY373
PRY494.1	PRY492.1	YSSH211.3
PRY496.7	PRY495.7	PRY373
PRY497.7	PRY495.7	YSSH211.3
PRY501.5	PRY500.5	YSSH211.3
PRY517.2	PRY516.2	YSSH211.3
PRY533.10	PRY532.10	YSSH211.3
PRY536	YSSH022	PRY373
PRY537	YSSH040	PRY373
YSSD224	YSSH175.7	YSSH211.3
YSSD225	YSSH183.1	YSSH211.3

Table S5. MAT α strain list

Strain Number	Strain Description	Origin/Parent
YSSH208	MAT α *MRP7::P_{MRP7}_GEV_HIS3 (5 copies)*	pPR1→ Y11044 BY4742
PRY373	MAT α *RET2::P_{RET2} TetR-SSN6_T_{ACT1}_URA3 , MRP7::P_{MRP7}_GEV_HIS3 (5 copies)*	pPR4→ YSSH208
YSSH211.3	MAT α *RET2::P_{RET2}_TetR-SIR3_T_{ACT1}_URA3, MRP7::P_{MRP7}_GEV_HIS3 (5 copies)*	pPR13→ YSSH208
YSSH25.4	MAT α *RET2::P_{MYO1} rtTA_T_{CYC1}_URA3, MRP7::P_{MRP7}_GEV_HIS3 (5 copies)*	→ YSSH208
PRY395.6	MAT α *hda1::LEU2, MRP7::P_{MRP7}_GEV_HIS3 (5 copies)*	pPR93→ YSSH208
PRY395.8	MAT α *hda1::LEU2, MRP7::P_{MRP7}_GEV_HIS3 (5 copies)*	pPR93→ YSSH208
PRY396.7	MAT α *hda1::LEU2, RET2::P_{RET2} TetR-SSN6_T_{ACT1}_ URA3 , MRP7::P_{MRP7}_GEV_HIS3 (5 copies)*	pPR93→ PRY373
PRY396.8	MAT α *hda1::LEU2, RET2::P_{RET2} TetR-SSN6_T_{ACT1}_ URA3 , MRP7::P_{MRP7}_GEV_HIS3 (5 copies)*	pPR93→ PRY373
PRY465.5	MAT α *srb10::LEU2, MRP7::P_{MRP7}_GEV_HIS3 (5 copies)*	pPR101→ YSSH208
PRY466.1	MAT α *srb10::LEU2, RET2::P_{RET2} TetR-SSN6_T_{ACT1}_ URA3 , MRP7::P_{MRP7}_GEV_HIS3 (5 copies)*	pPR101→ PRY373
PRY466.3	MAT α *srb10::LEU2, RET2::P_{RET2}_TetR- SSN6_T_{ACT1}_ URA3 , MRP7::P_{MRP7}_GEV_HIS3 (5 copies)*	pPR101→ PRY373

Table S6. MAT A strain list

Strain Number	Strain Description	Origin/Parent
PRY002	MAT A *FIG1::I_{FIG1}_tetO7_P_{GAL1NR}_GFP_T_{CYC1} _URA3(1 copy)*	pPR2→ Y01044 BY4741
PRY033	MAT A *FIG1::I_{FIG1}_T_{GAL7}_P_{GAL1NR}_GFP_tetO4 _URA3(1 copy)*	pPR10→ Y01044 BY4741
PRY034	MAT A *FIG1::I_{FIG1}_T_{GAL7}_P_{GAL1NR}_GFP_tetO4 _URA3(2 copies)*	pPR10→ Y01044 BY4741
PRY043	MAT A *FIG1::I_{FIG1}_tetO7_P_{GAL1NR}_GFP_tetO4 _URA3(1 copy)*	pPR11→ Y01044 BY4741
PRY224	MAT A *FIG1::I_{FIG1}_tetO2_P_{GAL1NR}_[GFP]2_tetO4 _URA3(1 copy)*	pPR33→ Y01044 BY4741
PRY237	MAT A *FIG1::I_{FIG1}_T_{GAL7}_P_{GAL1NR}_[GFP]2_tetO4 _URA3(2 copies)*	pCF101→ Y01044 BY4741
PRY263	MAT A *DAN1::I_{DAN1}_T_{GAL7}_P_{GAL1NR}_GFP_ tetO4_URA3(1 copy)*	pPR46→ Y01044 BY4741
PRY264	MAT A *DAN1::I_{DAN1}_T_{GAL7}_P_{GAL1NR}_GFP_ tetO4_URA3(2 copies)*	pPR46→ Y01044 BY4741
PRY265	MAT A *DAN1::I_{DAN1}_T_{GAL7}_P_{GAL1NR}_GFP_ tetO4_URA3(2 copies)*	pPR46→ Y01044 BY4741
PRY283	MAT A *DAN1::I_{DAN1}_T_{GAL7}_P_{GAL1NR}_GFP_T_{ACT1}_YFP _tetO4_URA3(2 copies)*	pPR48→ Y01044 BY4741
PRY292	MAT A *DAN1::I_{DAN1}_T_{GAL7}_P_{GAL1NR}_[GFP]2_ tetO4 _URA3(2 copies)*	pCF4→ Y01044 BY4741
PRY397	MAT A *hda1::LEU2, YFR054C::$I_{YFR054C}$_ T_{ADH1}-tetO2 _ P_{GAL1NR}_ GFP_ T_{CYC1}_URA3(2 copies)*	pPR93→ YSSH162.5
PRY455	MAT A *FIG1::I_{FIG1}_T_{GAL7}_P_{GAL1NR}_GFP_tetO4 _URA3 (2 copies)*	pPR10→ Y01044 BY4741
PRY456	MAT A *FIG1::I_{FIG1}_T_{GAL7}_P_{GAL1NR}_GFP_tetO4 _URA3(2 copies)*	pPR10→ Y01044 BY4741
PRY467.1	MAT A *srb10::LEU2, YFR054C::$I_{YFR054C}$_ T_{ADH1}-tetO2 _ P_{GAL1NR} _GFP_T_{CYC1}_URA3(2 copies)*	pPR101→ YSSH162.5
PRY467.7	MAT A *srb10::LEU2, YFR054C::$I_{YFR054C}$_ T_{ADH1}-tetO2 _P_{GAL1NR}_GFP_T_{CYC1}_URA3(2 copies)*	pPR101→ YSSH162.5
PRY468.2	MAT A *srb10::LEU2, YFR054C::$I_{YFR054C}$_T_{GAL7}_ P_{GAL1NR}_GFP_tetO4_URA3(2-3 copies)*	pPR101→ YSSH167.1
PRY468.6	MAT A *srb10::LEU2, YFR054C::$I_{YFR054C}$_T_{GAL7}_ P_{GAL1NR}_GFP_tetO4_URA3(2-3 copies)*	pPR101→ YSSH167.1
PRY470.3	MAT A *FIG1::I_{FIG1}_T_{GAL7}_P_{GAL1NR}_[GFP]2_tetO4 _URA3(1 copy)*	pCF101→ Y01044 BY4741

PRY473.4	MAT A *YFR054C::$I_{YFR054C+ter}$_tetO2_ T_{GAL7}_P_{GAL1NR}_GFP _ T_{CYC1} _URA3(1 copy)*	pPR 102→ Y01044 BY4741
PRY476.1	MAT A *YFR054C::$I_{YFR054C+ter}$_tetO2_ T_{GAL7}_P_{GAL1NR}_GFP _ tetO4 _URA3(1 copy)*	pPR 103→ Y01044 BY4741
PRY479	MAT A *FIG1::I_{FIG1}_T_{GAL7}_P_{GAL1NR}_GFP_tetO4 _URA3(1 copy), MRP7::P_{MRP7}_GEV_HIS3 (5 copies)*	pPR1→ PRY033
PRY480.3	MAT A *FIG1::I_{FIG1}_ T_{ADH1}-tetO2 _P_{GAL1NR}_GFP_T_{CYC1} _URA3(1 copy) MRP7::P_{MRP7}_GEV_HIS3 (5 copies)*	pPR1→ YSSH074
PRY481.4	MAT A *FIG1::I_{FIG1}_T_{GAL7}_P_{GAL1NR}_GFP_tetO4 _URA3(1 copy), MRP7::P_{MRP7}_GEV_HIS3 (5 copies), RET2::P_{MYO2} rtTA_T_{CYC1}_LEU2*	pPR90→ PRY479
PRY482.3	MAT A *FIG1::I_{FIG1}_ T_{ADH1}-tetO2 _P_{GAL1NR}_GFP_T_{CYC1} _URA3(1 copy) MRP7::P_{MRP7}_GEV_HIS3 (5 copies), RET2::P_{MYO2} rtTA_T_{CYC1}_LEU2*	pPR90→ PRY480.3
PRY485.1	MAT A *YFR054C::$I_{YFR054C+ter}$_tetO2_Spacer B (300) _ T_{GAL7}_P_{GAL1NR}_ GFP _ T_{CYC1} _URA3(1 copy)*	pPR109→ Y01044 BY4741
PRY489.1	MAT A *YFR054C::$I_{YFR054C}$_T_{GAL7}_P_{GAL1NR}_lacZ(150)_ tetO4 _URA3 (1 copy)*	pPR111→ Y01044 BY4741
PRY492.1	MAT A *YFR054C::$I_{YFR054C}$_T_{GAL7}_P_{GAL1NR}_lacZ(450)_ tetO4 _URA3 (1 copy)*	pPR112→ Y01044 BY4741
PRY495.7	MAT A *IRC7::I_{IRC7}_T_{GAL7}_P_{GAL1NR}_GFP_ tetO4 _URA3 (1 copy)*	pPR115→ Y01044 BY4741
PRY500.5	MAT A *YFR054C:: :$I_{YFR054C}$_ T_{ADH1}-tetO2 _ P_{GAL1NR}_ [GFP]2 _ tetO4 _URA3(1 copy)*	pPR116→ Y01044 BY4741
PRY516.2	MAT A *FIG1::I_{FIG1}_ T_{ADH1}-tetO7 _P_{GAL1NR}_ GFP_T_{ACT1} _lacZ_tetO4 _URA3(1 copy)*	pPR126→ Y01044 BY4741
PRY532.10	MAT A *FIG1::I_{FIG1}_ T_{ADH1}-tetO7 _P_{GAL1NR}_ GFP_T_{CYC1} _E-element _URA3(1 copy)*	pPR6→ Y01044 BY4741
YSSH022	MAT A *FIG1::I_{FIG1}_ T_{ADH1}-tetO2 _P_{GAL1NR}_ GFP_T_{ACT1} _YFP_tetO4 _URA3(1 copy)*	pPR36→ Y01044 BY4741
YSSH040	MAT A *FIG1::I_{FIG1}_ T_{ADH1}-tetO2 _P_{GAL1NR}_GFP_T_{ACT1} _lacZ_tetO4 _URA3(1 copy)*	pPR37→ Y01044 BY4741
YSSH043	MAT A *FIG1::I_{FIG1}_ T_{ADH1}-tetO2 _P_{GAL1NR}_GFP_T_{ACT1} _lacZ_T_{CYC1} _URA3(1 copy)*	pPR35→ Y01044 BY4741
YSSH070	MAT A *FIG1::I_{FIG1}_tetO7_P_{GAL1NR}_GFP_T_{CYC1} _URA3 (2 copies)*	pPR2→ Y01044 BY4741
YSSH071	MAT A *FIG1::I_{FIG1}_ T_{ADH1}-tetO2 _P_{GAL1NR}_[GFP]2_T_{CYC1} _URA3(1 copy)*	pPR34→ Y01044 BY4741
YSSH074	MAT A *FIG1::I_{FIG1}_ T_{ADH1}-tetO2 _P_{GAL1NR}_GFP_T_{CYC1} _URA3(1 copy)*	pPR08→ Y01044 BY4741
YSSH075	MAT A *FIG1::I_{FIG1}_ T_{ADH1}-tetO2 _P_{GAL1NR}_GFP_T_{CYC1} _URA3(2 copies)*	pPR08→ Y01044 BY4741
YSSH077	MAT A *FIG1::I_{FIG1}_ T_{ADH1}-tetO2 _P_{GAL1NR}_GFP_tetO4 _URA3(1 copy)*	pPR33→ Y01044 BY4741
YSSH097	MAT A *BAT2::I_{BAT2}_T_{GAL7}_P_{GAL1NR}_GFP_tetO4 _URA3*	pPR54→ Y01044

	(2 copies)	BY4741
YSSH152.3	MAT A *YFR054C::$I_{YFR054C+ter}$_tetO2_Spacer B_T_{GAL7}_P_{GAL1NR}_GFP_T_{CYC1}_URA3 (1 copy)*	pPR68→ Y01044 BY4741
YSSH162.2	MAT A *YFR054C::$I_{YFR054C}$_ T_{ADH1}-tetO2 _P_{GAL1NR}_GFP_ T_{CYC1} _URA3 (1 copy)*	pPR70→ Y01044 BY4741
YSSH162.4	MAT A *YFR054C::$I_{YFR054C}$_ T_{ADH1}-tetO2 _P_{GAL1NR}_GFP_ T_{CYC1} _URA3(3 copies)*	pPR70→ Y01044 BY4741
YSSH162.5	MAT A *YFR054C::$I_{YFR054C}$_ T_{ADH1}-tetO2 _P_{GAL1NR}_GFP_ T_{CYC1} _URA3 (2 copies)*	pPR70→ Y01044 BY4741
YSSH167.1	MAT A *YFR054C::$I_{YFR054C}$_T_{GAL7}_P_{GAL1NR}_GFP_ tetO4 _URA3 (2-3 copies)*	pPR71→ Y01044 BY4741
YSSH167.3	MAT A *YFR054C::$I_{YFR054C}$ _T_{GAL7}_P_{GAL1NR}_ GFP_tetO4 _URA3(1 copy)*	pPR71→ Y01044 BY4741
YSSH167.6	MAT A *YFR054C::$I_{YFR054C}$_T_{GAL7}_P_{GAL1NR} _GFP_tetO4 _URA3(3 copies)*	pPR71→ Y01044 BY4741
YSSH168.4	MAT A *YFR054C::$I_{YFR054C}$_ T_{ADH1}-tetO2 _P_{GAL1NR}_GFP _tetO4_URA3(1 copy)*	pPR74→ Y01044 BY4741
YSSH169.10	MAT A *BAT2::I_{BAT2}_T_{GAL7}_P_{GAL1NR}_[GFP]2 _tetO4_URA3 (2 copies)*	pCF03→ Y01044 BY4741
YSSH175.7	MAT A *YFR054C::$I_{YFR054C}$_ T_{ADH1}-tetO2 _P_{GAL1NR}_GFP_ T_{ACT1}_YFP_tetO4 _URA3(1 copy)*	PPR78→ Y01044 BY4741
YSSH183.1	MAT A *YFR054C::$I_{YFR054C}$_T_{GAL7}_P_{GAL1NR}_GFP _T_{ACT1} _lacZ_tetO4 _URA3(1 copy)*	pPR80→ Y01044 BY4741
YSSH184.2	MAT A *YFR054C::$I_{YFR054C}$_T_{GAL7}_P_{GAL1NR}_GFP _T_{ACT1} _YFP_tetO4 _URA3(1 copy)*	pPR81→ Y01044 BY4741
YSSH185.1	MAT A *YFR054C::$I_{YFR054C}$_T_{GAL7}_P_{GAL1NR}_[GFP]2 _tetO4 _URA3(1 copy)*	pPR82→ Y01044 BY4741
YSSH185.2	MAT A *YFR054C::$I_{YFR054C}$_T_{GAL7}_P_{GAL1NR}_[GFP]2 _tetO4_URA3 (2 copies)*	pPR82→ Y01044 BY4741
YSSH186.1	MAT A *YFR054C::$I_{YFR054C}$_T_{GAL7}_P_{GAL1NR}_GFP _T_{ACT1} _lacZ_tetO4 _URA3(1 copy)*	pPR80→ Y01044 BY4741
YSSH194.1	MAT A *YFR054C::$I_{YFR054C+ter}$_tetO2_Spacer B _ T_{GAL7}_P_{GAL1NR}_ GFP _ tetO4 _URA3(1 copy)*	pPR88→ Y01044 BY4741
YSSH214.3	MAT A *DAN1::I_{DAN1}_T_{GAL7}_P_{GAL1NR}_GFP_T_{ACT1} _lacZ_tetO4 _URA3 (2 copies)*	pPR92→ Y01044 BY4741
YSSH223.1	MAT A *hda1::LEU2, YFR054C::$I_{YFR054C}$_T_{GAL7}_ P_{GAL1NR}_GFP_tetO4 _URA3(2-3 copies)*	pPR93→ YSSH167.1
YSSH223.2	MAT A *hda1::LEU2, YFR054C::$I_{YFR054C}$_T_{GAL7}_ P_{GAL1NR}_GFP_tetO4 _URA3(2-3 copies)*	pPR93→ YSSH167.1

5. 2 Cooperation between activators

We observed interaction between upstream and downstream repressors and silencing proteins. We were interested in checking if there was cooperation between two different activators in dual activator recruiting construct and if there was any cooperation could it be broken by a repressor. The promoter was PGal1NR with upstream activator sequence of four GEV binding sites and downstream tet operators recruiting rtTA (Figure 3). It was observed that both the inducible activators did not cooperate with each other (Figure 5).

Figure 3: Reporter construct with GAL1UAS (orange box), GAL1TATA (black dotted box), GFP (green box), terminators (black arrows) and tet operators (blue boxes) that recruit rtTA.

We also used another construct with full Gal1 promoter (including Mig1 sites) PGal1 to study the effect of Mig1 on gene expression (Figure 2). We noticed that Mig1 had hardly any effect on gene expression though the strains were grown in glucose. As a control we grew the strains in raffinose there was no difference in inhibition but expression was 1.5 times higher in raffinose.

Figure 4: Reporter construct with GAL1Promoter (UAS-orange box, Mig1 binding sites-violet box, TATA-black dotted box), GFP (green box), terminators (black arrows) and tet operators (blue boxes) that recruit rtTA.

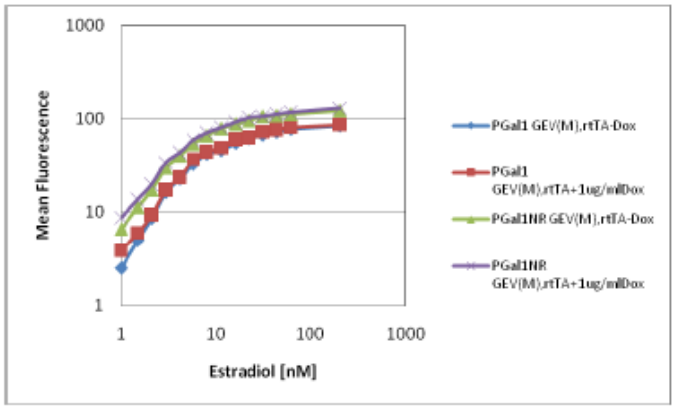

Figure 5: Effect of downstream activatior binding sites on gene expression.

Expression with increasing concentration of estradiol in the presence and absence of 2mM (1mg/ml) of doxycyclin. Doxycyclin induces binding of rtTA to tet operators.

5.3 Effect of downstream activator binding site on Silencing

Our previous results indicated a strong synergy between the interacting upstream and downstream silecning gradients in tetO-GFP-tetO construct. Cooperation of silencers is an endogenous feature of silencing and also variegated expression.

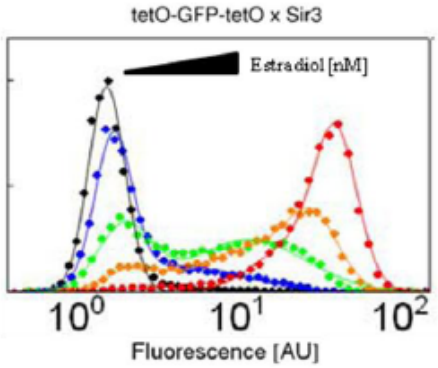

Figure 6: Cellular fluorescence distributions

The expression of tetO-GFP-tetO construct repressed by tetR-Sir3. The cells were induced by 3.75(black), 7.5 (blue), 15 (green), 30 (orange), and 200 (red) nM estradiol, in the absence of doxycycline.

In order to see the effect of a downstream activator binding site on the synergy and variegated expression in tetO-GFP-tetO construct we made a construct with activator binding sites are inserted between the terminator of the GFP and the tet operators (Figure 7). As a control activator binding sites are also inserted similarly in the GFP-tetO construct. The downstream activator binding sites did not contribute to activation of gene expression. The inhibition strength is reduced relative to the parent strain. The synergy of silencing gradients and bimodal expression shifted towards lower gene activation.

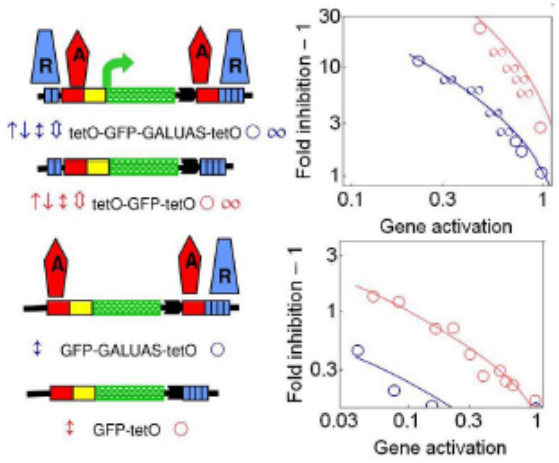

Figure 7: Effect of downstream activator on silencing.

Fold inhibition-1 of constructs with downstream GALUAS compared to that of construct without downstream GALUAS (O symbols are monostable and ∞ are bistable).

5.4 Effect of Ubp10 deletion on silencing

Ubp10, a ubiquitin protease is known to affect silencing both on deletion and overexpression (Singer M.S et al, Genetics, 1998; Kahana A and Gottschling D.E, Mol.Cell.Biol, 1999). Ubp10 regulates telomeric gene silencing through histone H2B deubiquitylation. Ubp10 is known to maintain low gene expression. Ubp10 interacts with Sir2 and Sir4 and mutually localizes with Sir proteins at telomere and telomere proximal regions. UBP10 deletion causes a 2- to 3-fold decrease in Sir2-HA levels at the telomere regions (Emre NC, Mol. Cell, 2005).

Basing on this concept we wanted to check if deletion of Ubp10 had any effect on silencing in our tetO-GFP-tetO construct at YFR054C locus, approximately 11 kb from the telomere. We also checked the effect of Ubp10 deletion on tetO-GFP and GFP-tetO constructs. We observed that silencing fold increases on deletion of Ubp10 in tetO-GFP-tetO construct only at lower activation conditions. Ubp10 deletion no effect on tetO-GFP and GFP-tetO constructs (Figure 8).

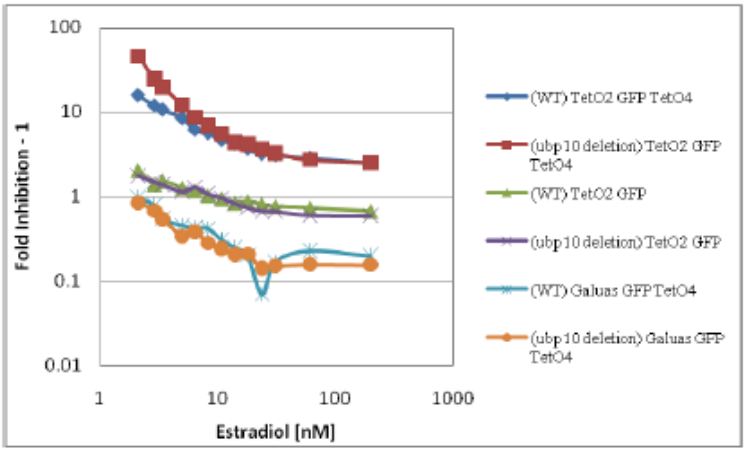

Figure 8: Effect of Ubp10 on silencing

Inhibition of gene expression expressed as fold inhibition-1 of tetO-GFP, GFP-tetO and tetO-GFP-tetO in wildtype (WT) and Ubp10 deleted strains.

5.5 Silencing contributes to noise in gene expression

Silencing proteins spread along the chromosome in a cooperative fashion and lead to heterochromatin formation. To score the cooperativity of tetR~Sir3, tetO-GFP-tetO construct was used. The cooperativity of tetR~Sir3 was calculated as Hill coefficient, which was 2.6 reflecting its Switch-like behavior (Figure 9). Hill coefficient is used here to measure Switch-like behavior, but does not reflect the cooperative fold increase in binding in absolute terms. The following formula was used to calculate the Hill coefficient

Log (x/1-x)=n log [D]

n= Hill coefficient

[D]= concentration of Dox

x: fraction of fluorescence at particular[D]

Previous studies implicated that transcriptional cooperativity leads to amplification of noise (A. Becskei, Nature Genetics, 2005). To evaluate the contribution of tetR~Sir3 we designed a two color experiment using YFP (Yellow Fluorescent Protein) and CFP (Cyan Fluorescent Protein) and integrated at YFR054C locus. A diploid strain with tetO-CFP-tetO and tetO-YFP-tetO constructs both integrated at YFR054C on the homologous chromosomes was studied using AxioVison 4.6 software. Fluctuations were observed in YFP and CFP correlated expression (Figure 10). Some cells had stronger CFP expression than YFP expression and vice versa. The cooperativity of tetR-Sir3 might contribute to the correlated noise. The uncorrelated noise could be because of the differential spatial localization of the homologous chromosomes (Table 4).

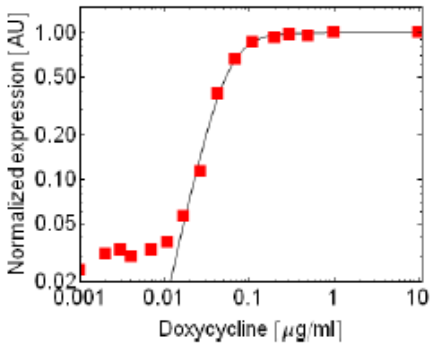

Figure 9: Cooperativity of tetR~Sir3

Switch-like behavior measured with Hill coefficient of tetR~Sir3.

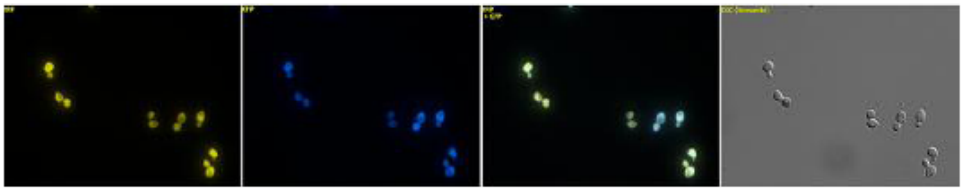

Figure 10: Co-expression of CFP and YFP.

DIC image and Fluorescence images of YFP alone, CFP alone and overlay of YFP and CFP measurements images of dipoid cells expressing [tetO]7-CFP-[tetO]4 and [tetO]7-YFP-[tetO]4 regulated by tetR-Sir3p. Cells were induced by estradiol at 64 nM and 0.08 μM of doxycycline.

Estradiol (nM)	200	64	200
Dox (microg/ml)	0.039	1	1
Total Noise of CFP Expression	0.68	0.53	0.27
Total Noise of YFP Expression	0.57	0.39	0.18
Correlated Noise	0.51	0.39	0.16
Uncorrelated Noise	0.47	0.35	0.19

Table 4: Noise in gene expression due to Sir3

5.6 Nucleation lengths determine bistability

Our preliminary data suggested that tetO2-GFP-teO4 has stronger silencing than tetO7-GFP and exhibited bimodality. This has initiated us to explore the effect of nucleation architecture on silencing. When the number of downstream nucleation sites is halved in tetO2-GFP-teO4, the resultant construct tetO2-GFP-teO2 showed a bistable response. In the construct tetO1-GFP-teO2 obtained by reducing the number of both upstream and downstream nucleation sites to half of tetO2-GFP-teO4, a bistable response prevailed. The construct with single nucleation sites upstream and downstream, tetO1-GFP-tetO1 construct displayed weak silencing and monostable gene expression indicating that synergistic interaction of gradients occurs only when the nucleation widths reach a certain threshold (Figure 11).

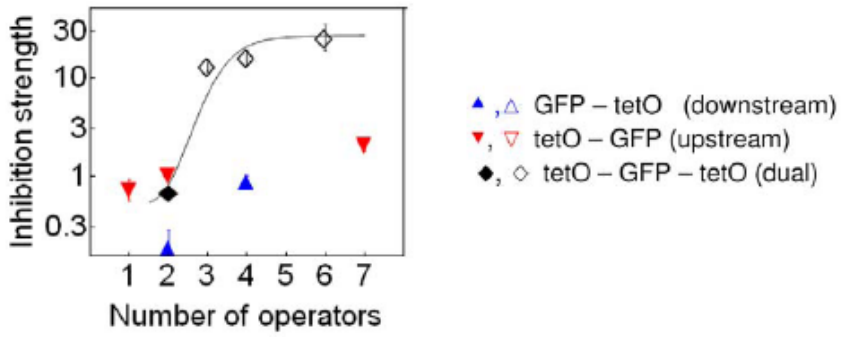

Figure 11: Inhibition strength at single (upstream or downstream) and dual recruitment constructs.

The inhibition strength is the average value for fold inhibition 21 in the [0.06, 0.6] interval of GA. The total number of tet operators is indicated for each dual recruitment construct [tetO]1-GFP-[tetO]1 (n= 2), [tetO]1-GFP-[tetO]2 (n = 3), [tetO]2-GFP-[tetO]2 (n = 4), and [tetO]2-GFP-[tetO]4 (n = 6).Empty symbols stand for constructs that display bimodal gene expression.

5.7 Silencing with I-silencer of HML locus

The HML mating type locus is flanked by E and I silencers which cooperate over a distance of 4000 bp (Boscheron C et al, EMBO J, 1996). They cannot inhibit gene expression individually. Silencing nucleated by tetR~Sir3 and E-silencer are similar. The constructs tetO-GFP-E and tetO-GFP-tetO behave similarly with respect to synergy. E-silencer alone did not inhibit gene expression (Ratna P et al, J. Mol. Biol, 2009). Similar to the earlier results I-silencer alone does not repress the reporter gene (Figure 12). The expression profile shown is in the presence of 2μM doxycyclin, where tetR~Sir3 does not bind to the tetO. Even with tetR~Sum1-1, the reponse is similar. It somehow has rather a weak activatory potential as the reporter gene had higher fluorescence in construct with I-silencer.

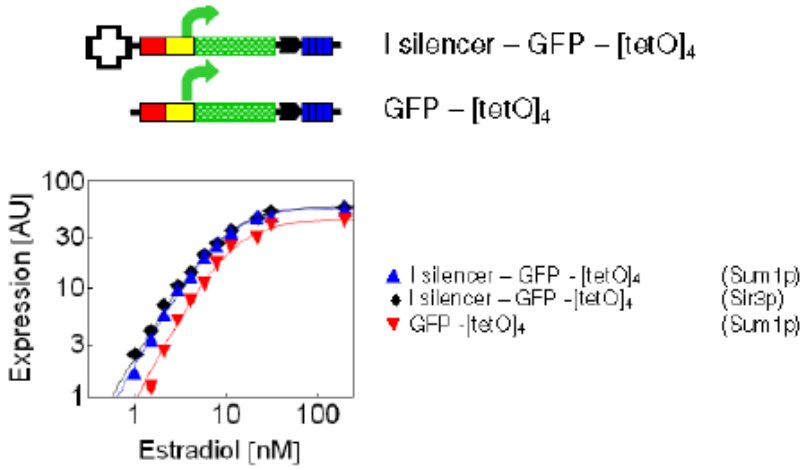

Figure 12: The I-silencer alone does not repress the reporter gene.

The expression induced by GEV at the I silencer-GFP-[tetO]4 construct was not lower than that at the GFP – tetO4 construct, in nonrepressive conditions. tetR-Sum1p and tetR-Sir3p do not repress expression in the presence of 2 μM doxycycline. Thus, the I-silencer alone does not repress the reporter gene; it has rather a weak activatory potential.

5.8 Silencing with HML silencers at YFR054C

Silencing was observed in tetO-GFP-tetO and I-silencer GFP-tetO constructs at YFR054C on recruiting tetR~Sir3. HML silencers (E-silencer & I-silencer) recruited SIR proteins leading to compaction of chromatin. To test the silencing strength of HML silencer, we made promoter-reporter construct flanked by HML silencers and integrated at YFR054C locus. We did not observe any silencing with I-silencer-GFP-E-silencer at YFR054C locus which was in accordance with the earlier work where silencing with HML silencers was studied at different loci (LYS2, HIS3, KEX2 and SIN4) (Maillet L et al, Genes and Development,1996). tetO-GFP-tetO also displayed chromosome position effect with inducible recruitment of tetR~Sir3.

5.9 Silencing in Centromeric Plasmid constructs

An earlier work showed that insertion of HML::LEU2-lacZ DNA into a CEN-containing plasmid inhibited gene expression to the same extent, as observed at the native locus (Maillet L et al, Genes and Development, 1996). We cloned the tetO-GFP-tetO constructs into a CEN-containing plasmid and compared its inhibition with that of the YFR054C locus. The fluorescence was measured at 4 hours, 8 hours and 24 hours after induction of gene expression.

Interestingly, the silencing strength of tetO-GFP-tetO was similar both in the CEN-containing plasmid and at YFR054C locus and the silencing strength remained the same over a period of time. Bimodal expression, a feature of silencing, was observed. There were two different kinds of population: 'On' (GFP expressing) and 'Off' (GFP non-expressing). The percent 'On' cells increased over a period of time at YFR054C locus but not in the case of CEN-containing plasmid.

The silencing strength of I-silencer GFP-tetO at YFR054C was compared to CEN-containing I-silencer GFP-tetO plasmid. Silencing strength was higher in the CEN-containing plasmid than at the YFR054C. The percent 'On' cells increased over a period of time in both cases (27, 49, 58 at 4 h, 8 h, 24 h respectively) (Figure 13). There were two sub-populations in 'On' cells in the strain containing I-silencer GFP-tetO in the CEN-containing plasmid. In the strains without tetR~Sir3, silencing was not observed at YFR054C locus, unlike that of I-silencer GFP-tetO in the CEN-containing plasmid which showed bimodal expression with increasing percent 'On' cells over time.

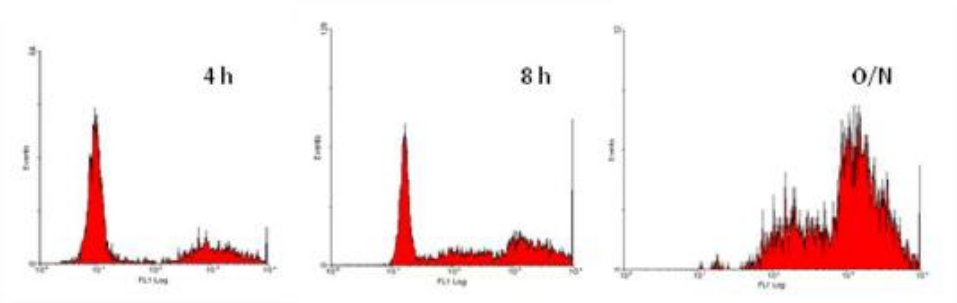

Figure 13: Mean distribution of cells expressing GFP.
Silencing at different time points of cells with GEV, tetR~Sir3 and CEN-containing I-silencer GFP-tetO.

To determine the silencing strength of HML silencers and the extent of variegated expression we cloned our reporter construct flanked by HML silencers into CEN-containing plasmid. Silencing strength increased with time. Their expression pattern was a combination of bimodality and graded response. Over time the percent 'On' cells increased (27, 52, 77 at 4 h, 8 h, 24 h respectively), and the 'Off' cells showed a graded response (Figure 14).

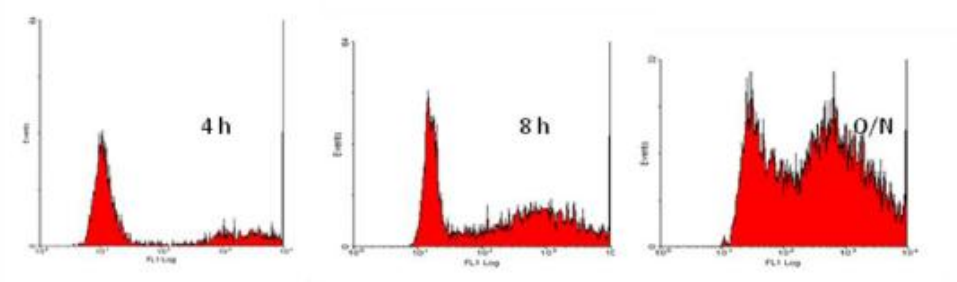

Figure 14: Mean distribution of cells expressing GFP.
Silencing at different time points of cells with GEV, tetR~Sir3 and CEN-containing I-silencer GFP- E-silencer.

Unfortunately, we were not successful in integrating our construct into the native HML locus.

5.10 Long range interactions between two different genes

The distribution of eukaryotic genes is known to be nonrandom and the essential genes cluster in persistently open chromatin domain (Batada N. N & Hurst L. D, 2007). Chromatin modification determines the expression patterns of adjacent genes (Batada N. N et al, 2007). To define the presence of any interactions between transcription factors of adjacent genes we chose two adjacent genes HXK1 and YFR054C. We integrated tetO-PGal1nr-lacZ construct towards centromere at HXK1 locus and PGal1nr-GFP construct towards telomere at YFR054C locus (Figure 15). As control we took strains that contained either of the constructs. We observed that tetR~Ssn6 bound to the tetO-PGal1nr-lacZ construct at HXK1 locus did not affect the GFP expression at YFR054C locus indicating that Ssn6 did not posses long range repression across adjacent genes.

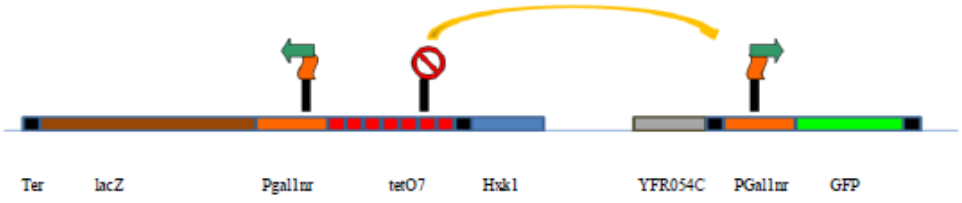

Figure 15: Depiction of Hxk1 tetO7-PGal1NR-lacZ and YFR054C PGal1NR-GFP constructs integrated into the genome.

6. DISCUSSION

The expression of eukaryotic genes requires the binding of transcriptional activators to their promoters and the subsequent recruitment of the RNA polymerase. Further regulation of transcription is attained by inhibitors of transcription. In this thesis, we aimed to gain an insight into repression and silencing phenomenon along the chromosome, to understand the underlying mechanisms and regulation of gene expression in budding yeast, *Saccharomyces cerevisiae*. We used synthetic constructs that could recruit inducible activators and inhibitors, to study the reciprocal relationship between the activators and repressors and to obtain a quantitative insight into gene expression.

Activators fail to activate gene expression when placed at 600-700 bases upstream or anywhere downstream of the gene (Keegan. L. et al, Science, 1986). A downstream activator is known to activate gene expression only when there is telomere looping (de Bruin, D. et al, 2001). Interaction between upstream and downstream activator binding sites is a question we wanted to address. In the dual inhibitory recruitment constructs, Ssn6 forms gradients and cooperates multiplicatively to oppose gene activation. Sir3 has shown synergetic amplification of silencing in dual inhibitory recruitment constructs. Such long range cooperativity between upstream and downstream activator binding sites is not observed in our findings (Figures 1-3). Unlike Ssn6 or Sir3, activators might not form long range gradients. The proximity of activator binding sites to the promoter determines the level of gene activation.

Transcriptional activators other than actuating gene expression, also dissuade the spreading of silencing proteins. The long-range effects of eukaryotic transcription factors are mostly mediated by chromatin modifying cofactors that spread along the chromatin. Activators procure chromatin modifiers that ease the chromatin structure curtailing diminishing the slope of the inhibitory concentration gradient (Fourel, G., C. Boscheron, et al. EMBO Rep, 2001). When we insert activator binding sites between the terminator of the GFP and the tet operators, we observe the synergy of silencing gradients and bimodal expression shifting towards lower gene activation (Figure 5). Though the downstream activator binding sites do not have any contribution to gene expression, binding of the activator alone seems to barricade the spreading of silencing proteins.

Silencing proteins physically interact with one another and with transcription factors bound to silencers and histones to establish heterochromatin. Following the nucleation, Sir2 deacytelates histones and enables formation of extended Sir protein complex along the chromatin. In vitro experiments have proved the highly cooperative binding interactions of Sir3 and Sir4, Hill coefficient being 3.1 (Daniel A.King et al, J. Biol. Chem, 2006). In our experiments, with the dual inhibitory recruitment construct we have calculated the cooperativity of Sir protein assembly in vivo. We have calculated the cooperativity of tetR~Sir3 as Hill coefficient, which is 2.6 reflecting its Switch-like behavior (Figure 8). The cooperativity of Ssn6 measured as Hill coefficient is 1.6. This cooperative action is one of the characteristic features of silencing proteins that makes it distinct from repressors. Variegated expression is yet another feature of silencing. We observe bimodality in constructs with dual recruitment sites with a certain nucleation threshold. Single recruitment construct close upstream to the promoter with seven operators exhibits monostability, whereas dual recruitment construct tetO2-GFP-tetO4 exhibits bimodality. Dual recruitment construct tetO1-GFP-tetO2 manifests bistability, but tetO1-GFP-tetO1 displays monostability. Thus cooperativity of silencing proteins is the threshold of nucleation sites which along with the architecture of nucleation sites determines variegated expression.

Cooperativity is also known to amplify noise in gene expression (A. Becskei, Nature Genetics, 2005). When similar dual recruitment constructs, one with CFP and the other with YFP, are integrated at YFR054C on the homologous chromosomes different kinds of population, some strongly expressing YFP, some CFP and some expressing both reporter genes similarly are observed. The cooperativity of Sir3 might contribute to different population, due to noise in gene expression. The noise could be because of the differential spatial localization of the homologous chromosomes in the nucleus.

Silencing is pertained to telomeres and HM loci on chromosome III. The HML mating type locus is flanked by E and I silencers which cooperate over a distance of 4000 bp. They do not inhibit gene expression when integrate at different loci or individually (Boscheron C et al, EMBO J, 1996; Maillet L et al, Genes and Development,1996). Similar to the earlier results, E and I silencers of HML, individually have not inhibited gene expression in our constructs. We have observed that when combined E and I- silencer with inducible recruitment sites, tetO-GFP-E-silencer and I- silencer-GFP-tetO, they manifested silencing and bimodality. We have not

observed any inhibition of gene expression when I-silencer-GFP-E-silencer when integrated at YFR054C. When we have cloned the I-silencer-GFP-E-silencer in a centromeric plasmid, E and I- silencers have interacted synergistically leading to silencing and variegated expression. We have observed a combination of bimodality and graded response in the expression pattern response (Figure 13), which reflects a complex synergy between E and I-silencers which is not understood. Centromeric plasmids are known to exist as nucleoprotein complexes in the nucleus (Seligy, VL et al, Nucleic Acids Research, 1980). They might associate with the nuclear envelop that is rich in Sir proteins, which explains the manifestation of silencing in I-silencer-GFP-E-silencer construct in centromeric plasmids.

The eukaryotic gene transcription inhibition mechanisms are not clear. Our research highlights a novel insight into repression and silencing, stating the similarities and differences in Ssn6 and Sir3 inhibitory gradients. Ssn6 and Sir3 have similar distance-dependent behavior. Silencing is different from repression when the binding sites flank the reporter gene. Ssn6 has a multiplicative effect on gene expression whereas Sir3 has strong synergistic effect. This synergistic effect is a result of the architecture of nucleation sites and cooperativity of silencing proteins. Knowledge of these inhibitory gradients help us understand the co-expression pattern of adjacent genes in the genome and the principles of cellular differentiation.

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8. REFERENCES

Andrulis, E. D., A. M. Neiman, et al. (1998). "Perinuclear localization of chromatin facilitates transcriptional silencing." Nature 394(6693): 592-5.

Ansari, A. and M. R. Gartenberg (1997). "The yeast silent information regulator Sir4p anchors and partitions plasmids." Mol Cell Biol 17(12): 7061-8.

Aparicio, O. M., B. L. Billington, et al. (1991). "Modifiers of position effect are shared between telomeric and silent mating-type loci in S. cerevisiae." Cell 66(6): 1279-87.

Arnold, S., M. Siemann, et al. (2001). "Kinetic modeling and simulation of in vitro transcription by phage T7 RNA polymerase." Biotechnol Bioeng 72(5): 548-61.

Balciunas, D. and H. Ronne (1995). "Three subunits of the RNA polymerase II mediator complex are involved in glucose repression." Nucleic Acids Res 23(21): 4421-5.

Becskei, A., B. B. Kaufmann, et al. (2005). "Contributions of low molecule number and chromosomal positioning to stochastic gene expression." Nat Genet 37(9): 937-44.

Bernstein, B. E., J. K. Tong, et al. (2000). "Genomewide studies of histone deacetylase function in yeast." Proc Natl Acad Sci U S A 97(25): 13708-13.

Bi, X., M. Braunstein, et al. (1999). "The yeast HML I silencer defines a heterochromatin domain boundary by directional establishment of silencing." Proc Natl Acad Sci U S A 96(21): 11934-9.

Bi, X. and J. R. Broach (1997). "DNA in transcriptionally silent chromatin assumes a distinct topology that is sensitive to cell cycle progression." Mol Cell Biol 17(12): 7077-87.

Bi, X. and J. R. Broach (2001). "Chromosomal boundaries in S. cerevisiae." Curr Opin Genet Dev 11(2): 199-204.

Bi, X., Q. Yu, et al. (2004). "Formation of boundaries of transcriptionally silent chromatin by nucleosome-excluding structures." Mol Cell Biol 24(5): 2118-31.

Borra, M. T., M. R. Langer, et al. (2004). "Substrate specificity and kinetic mechanism of the Sir2 family of NAD+-dependent histone/protein deacetylases." Biochemistry 43(30): 9877-87.

Boscheron, C., L. Maillet, et al. (1996). "Cooperation at a distance between silencers and proto-silencers at the yeast HML locus." EMBO J 15(9): 2184-95.

Brand, A. H., L. Breeden, et al. (1985). "Characterization of a "silencer" in yeast: a DNA sequence with properties opposite to those of a transcriptional enhancer." Cell 41(1): 41-8.

Carlson, M. (1997). "Genetics of transcriptional regulation in yeast: connections to the RNA polymerase II CTD." Annu Rev Cell Dev Biol 13: 1-23.

Carlson, M., B. C. Osmond, et al. (1984). "A suppressor of SNF1 mutations causesconstitutive high-level invertase synthesis in yeast." Genetics 107(1): 19-32.

Carmen, A. A., L. Milne, et al. (2002). "Acetylation of the yeast histone H4 N terminus regulates its binding to heterochromatin protein SIR3." J Biol Chem 277(7): 4778-81.

Carmen, A. A., S. E. Rundlett, et al. (1996). "HDA1 and HDA3 are components of a yeast histone deacetylase (HDA) complex." J Biol Chem 271(26): 15837-44.

Chen, G., J. Fernandez, et al. (1999). "A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in Drosophila development." Genes Dev 13(17): 2218-30.

Chen, L. and J. Widom (2005). "Mechanism of transcriptional silencing in yeast." Cell 120(1): 37-48.

Cheng, T. H., Y. C. Li, et al. (1998). "Persistence of an alternate chromatin structure at silenced loci in the absence of silencers." Proc Natl Acad Sci U S A 95(10): 5521-6.

Chi, M. H. and D. Shore (1996). "SUM1-1, a dominant suppressor of SIR mutations in Saccharomyces cerevisiae, increases transcriptional silencing at telomeres and HM mating-type loci and decreases chromosome stability." Mol Cell Biol 16(8): 4281-94.

Chien, C. T., S. Buck, et al. (1993). "Targeting of SIR1 protein establishes transcriptional silencing at HM loci and telomeres in yeast." Cell 75(3): 531-41.

Chou, C. C., Y. C. Li, et al. (2008). "Bypassing Sir2 and O-acetyl-ADP-ribose in transcriptional silencing." Mol Cell 31(5): 650-9.

Cockell, M. M., S. Perrod, et al. (2000). "Analysis of Sir2p domains required for rDNA and telomeric silencing in Saccharomyces cerevisiae." Genetics 154(3): 1069-83.

Cooper, J. P., S. Y. Roth, et al. (1994). "The global transcriptional regulators, SSN6 and TUP1, play distinct roles in the establishment of a repressive chromatin structure." Genes Dev 8(12): 1400-10.

Cranz, S., C. Berger, et al. (2004). "Monomeric and dimeric bZIP transcription factor GCN4 bind at the same rate to their target DNA site." Biochemistry 43(3): 718-27.

Cubizolles, F., F. Martino, et al. (2006). "A homotrimer-heterotrimer switch in Sir2 structure differentiates rDNA and telomeric silencing." Mol Cell 21(6): 825-36.

Davie, J. K., R. J. Trumbly, et al. (2002). "Histone-dependent association of Tup1-Ssn6 with repressed genes in vivo." Mol Cell Biol 22(3): 693-703.

de Bruin, D., S. M. Kantrow, et al. (2000). "Telomere folding is required for the stable maintenance of telomere position effects in yeast." Mol Cell Biol 20(21): 7991-8000.

de Bruin, D., Z. Zaman, et al. (2001). "Telomere looping permits gene activation by a downstream UAS in yeast." Nature 409(6816): 109-13.

Deckert, J., A. M. Torres, et al. (1998). "The anatomy of a hypoxic operator in Saccharomyces cerevisiae." Genetics 150(4): 1429-41.

Dhillon, N. and R. T. Kamakaka (2002). "Breaking through to the other side: silencers and barriers." Curr Opin Genet Dev 12(2): 188-92.

Donze, D., C. R. Adams, et al. (1999). "The boundaries of the silenced HMR domain in Saccharomyces cerevisiae." Genes Dev 13(6): 698-708.

Ducker, C. E. and R. T. Simpson (2000). "The organized chromatin domain of the repressed yeast a cell-specific gene STE6 contains two molecules of the corepressor Tup1p per nucleosome." EMBO J 19(3): 400-9.

Edmondson, D. G., M. M. Smith, and S. Y. Roth. (1996). "Repression domain of the yeast global repressor Tup1 interacts directly with histones H3 and H4." Genes Dev. 10: 1247–1259.

Edmondson, D. G., W. Zhang, et al. (1998). "In vivo functions of histone acetylation/deacetylation in Tup1p repression and Gcn5p activation." Cold Spring Harb Symp Quant Biol 63: 459-68.

Emre, N. C., K. Ingvarsdottir, et al. (2005). "Maintenance of low histone ubiquitylation by Ubp10 correlates with telomere-proximal Sir2 association and gene silencing." Mol Cell 17(4): 585-94.

Fleming, A. B. and S. Pennings (2001). "Antagonistic remodelling by Swi-Snf and Tup1-Ssn6 of an extensive chromatin region forms the background for FLO1 gene regulation." EMBO J 20(18): 5219-31.

Fourel, G., C. Boscheron, et al. (2001). "An activation-independent role of transcription factors in insulator function." EMBO Rep 2(2): 124-32.

Gavin, I. M. and R. T. Simpson (1997). "Interplay of yeast global transcriptional regulators Ssn6p-Tup1p and Swi-Snf and their effect on chromatin structure." EMBO J 16(20): 6263-71.

Georgel, P. T., M. A. Palacios DeBeer, et al. (2001). "Sir3-dependent assembly of supramolecular chromatin structures in vitro." Proc Natl Acad Sci U S A 98(15): 8584-9.

Ghidelli, S., D. Donze, et al. (2001). "Sir2p exists in two nucleosome-binding complexes with distinct deacetylase activities." EMBO J 20(16): 4522-35.

Gilson, E., M. Roberge, et al. (1993). "Distortion of the DNA double helix by RAP1 at silencers and multiple telomeric binding sites." J Mol Biol 231(2): 293-310.

Gottschling, D. E., O. M. Aparicio, et al. (1990). "Position effect at S. cerevisiae telomeres: reversible repression of Pol II transcription." Cell 63(4): 751-62.

Grbavec, D., R. Lo, et al. (1999). "Groucho/transducin-like enhancer of split (TLE) family members interact with the yeast transcriptional co-repressor SSN6 and mammalian SSN6-related proteins: implications for evolutionary conservation of transcription repression mechanisms." Biochem J 337 (Pt 1): 13-7.

Green, S. R. and A. D. Johnson (2004). "Promoter-dependent roles for the Srb10 cyclin-dependent kinase and the Hda1 deacetylase in Tup1-mediated repression in Saccharomyces cerevisiae." Mol Biol Cell 15(9): 4191-202.

Greenblatt, J. (1997). "RNA polymerase II holoenzyme and transcriptional regulation." Curr Opin Cell Biol 9(3): 310-9.

Grewal, S. I. and D. Moazed (2003). "Heterochromatin and epigenetic control of gene expression." Science 301(5634): 798-802.

Grozinger, C. M., C. A. Hassig, et al. (1999). "Three proteins define a class of human histone deacetylases related to yeast Hda1p." Proc Natl Acad Sci U S A 96(9): 4868-73.

Hanna-Rose, W. and U. Hansen (1996). "Active repression mechanisms of eukaryotic transcription repressors." Trends Genet 12(6): 229-34.

Hebbes, T. R., A. W. Thorne, et al. (1992). "Histone acetylation and globin gene switching." Nucleic Acids Res 20(5): 1017-22.

Hebbes, T. R., A. W. Thorne, et al. (1988). "A direct link between core histone acetylation and transcriptionally active chromatin." EMBO J 7(5): 1395-402.

Hecht, A., T. Laroche, et al. (1995). "Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: a molecular model for the formation of heterochromatin in yeast." Cell 80(4): 583-92.

Hecht, A., S. Strahl-Bolsinger, et al. (1996). "Spreading of transcriptional repressor SIR3 from telomeric heterochromatin." Nature 383(6595): 92-6.

Hengartner, C. J., C. M. Thompson, et al. (1995). "Association of an activator with an RNA polymerase II holoenzyme." Genes Dev 9(8): 897-910.

Herschbach, B. M., M. B. Arnaud, et al. (1994). "Transcriptional repression directed by the yeast alpha 2 protein in vitro." Nature 370(6487): 309-11.

Hild, M. and R. Paro (2003). "Anti-silencing from the core: a histone H2A variant protects euchromatin." Nat Cell Biol 5(4): 278-80.

Holmes, S. G. and J. R. Broach (1996). "Silencers are required for inheritance of the repressed state in yeast." Genes Dev 10(8): 1021-32.

Hoppe, G. J., J. C. Tanny, et al. (2002). "Steps in assembly of silent chromatin in yeast: Sir3-independent binding of a Sir2/Sir4 complex to silencers and role for Sir2-dependent deacetylation." Mol Cell Biol 22(12): 4167-80.

Huang, L., W. Zhang, et al. (1997). "Amino termini of histones H3 and H4 are required for a1-alpha2 repression in yeast." Mol Cell Biol 17(11): 6555-62.

Huang, M., Z. Zhou, et al. (1998). "The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor." Cell 94(5): 595-605.

Jackson, M. D. and J. M. Denu (2002). "Structural identification of 2'- and 3'-O-acetyl-ADP-ribose as novel metabolites derived from the Sir2 family of beta -NAD+-dependent histone/protein deacetylases." J Biol Chem 277(21): 18535-44.

Johnson, A., G. Li, et al. (2009). "Reconstitution of heterochromatin-dependent transcriptional gene silencing." Mol Cell 35(6): 769-81.

Kahana, A. and D. E. Gottschling (1999). "DOT4 links silencing and cell growth in Saccharomyces cerevisiae." Mol Cell Biol 19(10): 6608-20.

Kahana, A. and D. E. Gottschling (1999). "DOT4 links silencing and cell growth in Saccharomyces cerevisiae." Mol Cell Biol 19(10): 6608-20.

Kahana, A. and D. E. Gottschling (1999). "DOT4 links silencing and cell growth in Saccharomyces cerevisiae." Mol Cell Biol 19(10): 6608-20.

Kasten, M. M., S. Dorland, et al. (1997). "A large protein complex containing the yeast Sin3p and Rpd3p transcriptional regulators." Mol Cell Biol 17(8): 4852-8.

Kayne, P. S., U. J. Kim, et al. (1988). "Extremely conserved histone H4 N terminus is dispensable for growth but essential for repressing the silent mating loci in yeast." Cell 55(1): 27-39.

Keegan, L., G. Gill, et al. (1986). "Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein." Science 231(4739): 699-704.

Keleher, C. A., M. J. Redd, et al. (1992). "Ssn6-Tup1 is a general repressor of transcription in yeast." Cell 68(4): 709-19.

Khanin, R., V. Vinciotti, et al. (2007). "Statistical reconstruction of transcription factor activity using Michaelis-Menten kinetics." Biometrics 63(3): 816-23.

Kimura, A., T. Umehara, et al. (2002). "Chromosomal gradient of histone acetylation established by Sas2p and Sir2p functions as a shield against gene silencing." Nat Genet 32(3): 370-7.

King, D. A., B. E. Hall, et al. (2006). "Domain structure and protein interactions of the silent information regulator Sir3 revealed by screening a nested deletion library of protein fragments." J Biol Chem 281(29): 20107-19.

Kingston, R. E., C. A. Bunker, et al. (1996). "Repression and activation by multiprotein complexes that alter chromatin structure." Genes Dev 10(8): 905-20.

Klar, A. J., S. N. Kakar, et al. (1985). "SUM1, an apparent positive regulator of the cryptic mating-type loci in Saccharomyces cerevisiae." Genetics 111(4): 745-58.

Knott, S. R., C. J. Viggiani, et al. (2009). "Genome-wide replication profiles indicate an expansive role for Rpd3L in regulating replication initiation timing or efficiency, and reveal genomic loci of Rpd3 function in Saccharomyces cerevisiae." Genes Dev **23**(9): 1077-90.

Koleske, A. J. and R. A. Young (1994). "An RNA polymerase II holoenzyme responsive to activators." Nature 368(6470): 466-9.

Komachi, K., M. J. Redd, et al. (1994). "The WD repeats of Tup1 interact with the homeo domain protein alpha 2." Genes Dev 8(23): 2857-67.

Kuchin, S. and M. Carlson (1998). "Functional relationships of Srb10-Srb11 kinase, carboxy-terminal domain kinase CTDK-I, and transcriptional corepressor Ssn6-Tup1." Mol Cell Biol 18(3): 1163-71.

Kuchin, S., P. Yeghiayan, et al. (1995). "Cyclin-dependent protein kinase and cyclin homologs SSN3 and SSN8 contribute to transcriptional control in yeast." Proc Natl Acad Sci U S A 92(9): 4006-10.

Kyrion, G., K. Liu, et al. (1993). "RAP1 and telomere structure regulate telomere position effects in Saccharomyces cerevisiae." Genes Dev 7(7A): 1146-59.

Laurenson, P. and J. Rine (1991). "SUM1-1: a suppressor of silencing defects in Saccharomyces cerevisiae." Genetics 129(3): 685-96.

Lee, J. H., K. Maskos, et al. (2009). "Structural and functional studies of the yeast class II Hda1 histone deacetylase complex." J Mol Biol 391(4): 744-57.

Lee, J. M. and A. L. Greenleaf (1997). "Modulation of RNA polymerase II elongation efficiency by C-terminal heptapeptide repeat domain kinase I." J Biol Chem 272(17): 10990-3.

Lee, M., S. Chatterjee, et al. (2000). "Genetic analysis of the role of Pol II holoenzyme components in repression by the Cyc8-Tup1 corepressor in yeast." Genetics 155(4): 1535-42.

Li, B. and J. C. Reese (2001). "Ssn6-Tup1 regulates RNR3 by positioning nucleosomes and affecting the chromatin structure at the upstream repression sequence." J Biol Chem 276(36): 33788-97.

Liao, S. M., J. Zhang, et al. (1995). "A kinase-cyclin pair in the RNA polymerase II holoenzyme." Nature 374(6518): 193-6.

Liou, G. G., J. C. Tanny, et al. (2005). "Assembly of the SIR complex and its regulation by O-acetyl-ADP-ribose, a product of NAD-dependent histone deacetylation." Cell 121(4): 515-27.

Lu, Q., L. L. Wallrath, et al. (1994). "Nucleosome positioning and gene regulation." J Cell Biochem 55(1): 83-92.

Lynch, P. J., H. B. Fraser, et al. (2005). "Sum1p, the origin recognition complex, and the spreading of a promoter-specific repressor in Saccharomyces cerevisiae." Mol Cell Biol 25(14): 5920-32.

Lynch, P. J. and L. N. Rusche (2009). "A silencer promotes the assembly of silenced chromatin independently of recruitment." Mol Cell Biol 29(1): 43-56.

Mahoney, D. J. and J. R. Broach (1989). "The HML mating-type cassette of Saccharomyces cerevisiae is regulated by two separate but functionally equivalent silencers." Mol Cell Biol 9(11): 4621-30.

Maillet, L., C. Boscheron, et al. (1996). "Evidence for silencing compartments within the yeast nucleus: a role for telomere proximity and Sir protein concentration in silencer-mediated repression." Genes Dev 10(14): 1796-811.

Marcand, S., S. W. Buck, et al. (1996). "Silencing of genes at nontelomeric sites in yeast is controlled by sequestration of silencing factors at telomeres by Rap 1 protein." Genes Dev 10(11): 1297-309.

Micklem, G., A. Rowley, et al. (1993). "Yeast origin recognition complex is involved in DNA replication and transcriptional silencing." Nature 366(6450): 87-9.

Moretti, P., K. Freeman, et al. (1994). "Evidence that a complex of SIR proteins interacts with the silencer and telomere-binding protein RAP1." Genes Dev 8(19): 2257-69.

Moretti, P. and D. Shore (2001). "Multiple interactions in Sir protein recruitment by Rap1p at silencers and telomeres in yeast." Mol Cell Biol 21(23): 8082-94.

Mukai, Y., E. Matsuo, et al. (1999). "Conservation of histone binding and transcriptional repressor functions in a Schizosaccharomyces pombe Tup1p homolog." Mol Cell Biol 19(12): 8461-8.

Palaparti, A., A. Baratz, et al. (1997). "The Groucho/transducin-like enhancer of split transcriptional repressors interact with the genetically defined amino-terminal silencing domain of histone H3." J Biol Chem 272(42): 26604-10.

Park, E. C. and J. W. Szostak (1990). "Point mutations in the yeast histone H4 gene prevent silencing of the silent mating type locus HML." Mol Cell Biol 10(9): 4932-4.

Pillus, L. and J. Rine (1989). "Epigenetic inheritance of transcriptional states in S. cerevisiae." Cell 59(4): 637-47.

Redd, M. J., M. B. Arnaud, et al. (1997). "A complex composed of tup1 and ssn6 represses transcription in vitro." J Biol Chem 272(17): 11193-7.

Redd, M. J., M. R. Stark, et al. (1996). "Accessibility of alpha 2-repressed promoters to the activator Gal4." Mol Cell Biol 16(6): 2865-9.

Renauld, H., O. M. Aparicio, et al. (1993). "Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by SIR3 dosage." Genes Dev 7(7A): 1133-45.

Rine, J. and I. Herskowitz (1987). "Four genes responsible for a position effect on expression from HML and HMR in Saccharomyces cerevisiae." Genetics 116(1): 9-22.

Ronen, M., R. Rosenberg, et al. (2002). "Assigning numbers to the arrows: parameterizing a gene regulation network by using accurate expression kinetics." Proc Natl Acad Sci U S A 99(16): 10555-60.

Rossi, F. M., A. M. Kringstein, et al. (2000). "Transcriptional control: rheostat converted to on/off switch." Mol Cell 6(3): 723-8.

Roth, S. Y. (1995). "Chromatin-mediated transcriptional repression in yeast." Curr Opin Genet Dev 5(2): 168-73.

Roth, S. Y., J. M. Denu, et al. (2001). "Histone acetyltransferases." Annu Rev Biochem 70: 81-120.

Rundlett, S. E., A. A. Carmen, et al. (1996). "HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription." Proc Natl Acad Sci U S A 93(25): 14503-8.

Rusche, L. N., A. L. Kirchmaier, et al. (2003). "The establishment, inheritance, and function of silenced chromatin in Saccharomyces cerevisiae." Annu Rev Biochem 72: 481-516.

Rusche, L. N. and J. Rine (2001). "Conversion of a gene-specific repressor to a regional silencer." Genes Dev 15(8): 955-67.

Sauve, A. A., I. Celic, et al. (2001). "Chemistry of gene silencing: the mechanism of NAD+-dependent deacetylation reactions." Biochemistry 40(51): 15456-63.

Schultz, J., L. Marshall-Carlson, et al. (1990). "The N-terminal TPR region is the functional domain of SSN6, a nuclear phosphoprotein of Saccharomyces cerevisiae." Mol Cell Biol 10(9): 4744-56.

Sekinger, E. A. and D. S. Gross (2001). "Silenced chromatin is permissive to activator binding and PIC recruitment." Cell 105(3): 403-14.

Seligy, V. L., D. Y. Thomas, et al. (1980). "Saccharomyces cerevisiae plasmid, Scp or 2 mum: intracellular distribution, stability and nucleosomal-like packaging." Nucleic Acids Res 8(15): 3371-91.

Shea, M. A. and G. K. Ackers (1985). "The OR control system of bacteriophage lambda. A physical-chemical model for gene regulation." J Mol Biol 181(2): 211-30.

Shei, G. J. and J. R. Broach (1995). "Yeast silencers can act as orientation-dependent gene inactivation centers that respond to environmental signals." Mol Cell Biol 15(7): 3496-506.

Sikorski, R. S., M. S. Boguski, et al. (1990). "A repeating amino acid motif in CDC23 defines a family of proteins and a new relationship among genes required for mitosis and RNA synthesis." Cell 60(2): 307-17.

Simpson, R. T., S. Y. Roth, et al. (1993). "Nucleosome positioning and transcription." Cold Spring Harb Symp Quant Biol 58: 237-45.

Singer, M. S., A. Kahana, et al. (1998). "Identification of high-copy disruptors of telomeric silencing in Saccharomyces cerevisiae." Genetics 150(2): 613-32.

Smith, R. L. and A. D. Johnson (2000). "Turning genes off by Ssn6-Tup1: a conserved system of transcriptional repression in eukaryotes." Trends Biochem Sci 25(7): 325-30.

Smith, R. L., M. J. Redd, et al. (1995). "The tetratricopeptide repeats of Ssn6 interact with the homeo domain of alpha 2." Genes Dev 9(23): 2903-10.

Suka, N., Y. Suka, et al. (2001). "Highly specific antibodies determine histone acetylation site usage in yeast heterochromatin and euchromatin." Mol Cell 8(2): 473-9.

Sutton, A., R. C. Heller, et al. (2001). "A novel form of transcriptional silencing by Sum1-1 requires Hst1 and the origin recognition complex." Mol Cell Biol 21(10): 3514-22.

Svaren, J. and W. Horz (1997). "Transcription factors vs nucleosomes: regulation of the PHO5 promoter in yeast." Trends Biochem Sci 22(3): 93-7.

Szeto, L. and J. R. Broach (1997). "Role of alpha2 protein in donor locus selection during mating type interconversion." Mol Cell Biol 17(2): 751-9.

Tanny, J. C., D. S. Kirkpatrick, et al. (2004). "Budding yeast silencing complexes and regulation of Sir2 activity by protein-protein interactions." Mol Cell Biol 24(16): 6931-46.

Taunton, J., C. A. Hassig, et al. (1996). "A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p." Science 272(5260): 408-11.

Thompson, C. M., A. J. Koleske, et al. (1993). "A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast." Cell 73(7): 1361-75.

Treitel, M. A., S. Kuchin, et al. (1998). "Snf1 protein kinase regulates phosphorylation of the Mig1 repressor in Saccharomyces cerevisiae." Mol Cell Biol 18(11): 6273-80.

Triolo, T. and R. Sternglanz (1996). "Role of interactions between the origin recognition complex and SIR1 in transcriptional silencing." Nature 381(6579): 251-3.

Trumbly, R. J. (1986). "Isolation of Saccharomyces cerevisiae mutants constitutive for invertase synthesis." J Bacteriol 166(3): 1123-7.

Tzamarias, D. and K. Struhl (1994). "Functional dissection of the yeast Cyc8-Tup1 transcriptional co-repressor complex." Nature 369(6483): 758-61.

Vallier, L. G. and M. Carlson (1994). "Synergistic release from glucose repression by mig1 and ssn mutations in Saccharomyces cerevisiae." Genetics 137(1): 49-54.

Varanasi, U. S., M. Klis, et al. (1996). "The Cyc8 (Ssn6)-Tup1 corepressor complex is composed of one Cyc8 and four Tup1 subunits." Mol Cell Biol 16(12): 6707-14.

Verdone, L., G. Camilloni, et al. (1996). "Chromatin remodeling during Saccharomyces cerevisiae ADH2 gene activation." Mol Cell Biol 16(5): 1978-88.

Wahi, M. and A. D. Johnson (1995). "Identification of genes required for alpha 2 repression in Saccharomyces cerevisiae." Genetics 140(1): 79-90.

Wahi, M., K. Komachi, et al. (1998). "Gene regulation by the yeast Ssn6-Tup1 corepressor." Cold Spring Harb Symp Quant Biol 63: 447-57.

Waterborg, J. H. (2000). "Steady-state levels of histone acetylation in Saccharomyces cerevisiae." J Biol Chem 275(17): 13007-11.

Watson, A. D., Edmondson, D. G., Bone, J. R., Mukai, Y., Yu, Y., Du, W., Stillman, D. J., and Roth, S. Y. (2000). "Ssn6–Tup1 interacts with class I histone deacetylases required for repression." Genes Dev 14: 2737–2744.

Watson, A. D., D. G. Edmondson, et al. (2000). "Ssn6-Tup1 interacts with class I histone deacetylases required for repression." Genes Dev 14(21): 2737-44.

Weiss, K. and R. T. Simpson (1998). "High-resolution structural analysis of chromatin at specific loci: Saccharomyces cerevisiae silent mating type locus HMLalpha." Mol Cell Biol 18(9): 5392-403.

West, A. G., M. Gaszner, et al. (2002). "Insulators: many functions, many mechanisms." Genes Dev 16(3): 271-88.

Williams, F. E. and R. J. Trumbly (1990). "Characterization of TUP1, a mediator of glucose repression in Saccharomyces cerevisiae." Mol Cell Biol 10(12): 6500-11.

Williams, F. E., U. Varanasi, et al. (1991). "The CYC8 and TUP1 proteins involved in glucose repression in Saccharomyces cerevisiae are associated in a protein complex." Mol Cell Biol 11(6): 3307-16.

Wolffe, A. P. (1996). "Histone deacetylase: a regulator of transcription." Science 272(5260): 371-2.

Wu, J., A. A. Carmen, et al. (2001). "HDA2 and HDA3 are related proteins that interact with and are essential for the activity of the yeast histone deacetylase HDA1." Proc Natl Acad Sci U S A 98(8): 4391-6.

Xie, J., M. Pierce, et al. (1999). "Sum1 and Hst1 repress middle sporulation-specific gene expression during mitosis in Saccharomyces cerevisiae." EMBO J 18(22): 6448-54.

Yang, H. T., C. P. Hsu, et al. (2007). "An analytical rate expression for the kinetics of gene transcription mediated by dimeric transcription factors." J Biochem 142(2): 135-44.

Zaman, Z., A. Z. Ansari, et al. (2001). "Interaction of a transcriptional repressor with the RNA polymerase II holoenzyme plays a crucial role in repression." Proc Natl Acad Sci U S A 98(5): 2550-4.

Zhang, W., J. R. Bone, et al. (1998). "Essential and redundant functions of histone acetylation revealed by mutation of target lysines and loss of the Gcn5p acetyltransferase." EMBO J 17(11): 3155-67.

Zhang, Z. and A. R. Buchman (1997). "Identification of a member of a DNA-dependent ATPase family that causes interference with silencing." Mol Cell Biol 17(9): 5461-72.

Zhang, Z. and J. C. Reese (2004). "Redundant mechanisms are used by Ssn6-Tup1 in repressing chromosomal gene transcription in Saccharomyces cerevisiae." J Biol Chem 279(38): 39240-50.

Zhang, Z. and J. C. Reese (2004). "Ssn6-Tup1 requires the ISW2 complex to position nucleosomes in Saccharomyces cerevisiae." EMBO J 23(11): 2246-57.

Zhou, J., B. O. Zhou, et al. (2009). "Histone deacetylase Rpd3 antagonizes Sir2-dependent silent chromatin propagation." Nucleic Acids Res 37(11): 3699-713.

Zou, S., N. Ke, et al. (1996). "The Saccharomyces retrotransposon Ty5 integrates preferentially into regions of silent chromatin at the telomeres and mating loci." Genes Dev 10(5): 634-45.

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PAPERS PUBLISHED:

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5. Rajaram V, Prasad K, Ratna Prasuna P, Ramachandra N, Bharath SR, Savithri HS, Murthy MR. (2006) Cloning, purification, crystallization and preliminary X-ray crystallographic analysis of the biosynthetic N-acetylornithine aminotransferases from Salmonella typhimurium and Escherichia coli. Acta *Crystallograph Sect F Struct Biol Cryst Commun*. 62(Pt 10):980-3

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